SYNERGISTIC EFFECT OF IMIDACLOPRID ON CONIDIAL GERMINATION AND THE PATHOGENICITY OF TWO ENTOMOPATHOGENIC FUNGI TO LARVAE OF *Diaprepes abbreviatus* (COLEOPTERA: CURCULIONIDAE).

By

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Abstract of Dissertation Presented to the Graduate School
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SYNERGISTIC EFFECT OF IMIDACLOPRID ON CONIDIAL GERMINATION AND THE PATHOGENICITY OF TWO ENTOMOPATHOGENIC FUNGI TO LARVAE OF *Diaprepes abbreviatus* (COLEOPTERA: CURCULIONIDAE).

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December 1996

Chairperson: Dr. C. W. McCoy
Major Department: Entomology and Nematology

The effects of both sublethal and lethal doses of imidacloprid on *Diaprepes abbreviatus* first instar larval survival, ecdysis, and mobility using contact and oral exposure bioassays were determined as a precursor to further studies on the interaction of entomopathogenic fungi with sublethal doses of imidacloprid on larvae. At concentrations of imidacloprid at 12.5 ppm ai and greater, the chemical fed *per os* reduced larval mobility and feeding, which resulted in slower larval development and reduced ecdysis. Contact exposure to imidacloprid also affected larval mobility and development at doses greater than 100 ppm ai. Larval mobility was significantly impaired in soil treated with imidacloprid at doses greater than 6μg ai/g soil. Following contact exposure to the chemical, larvae recovered from imidacloprid-induced paralysis. Subsequent to oral exposure to imidacloprid without chemical, larvae recovered fully showing no sluggishness or quivering behavior.

Rate and number of conidia of *Metarhizium anisopliae* germinating on water agar increased 4-fold with an increase in imidacloprid concentration from 1 to 10,000 ppm ai. Imidacloprid applied to the larval cuticle also stimulated premature conidial germination. Conidial germination of both *M. anisopliae* and *Beauveria bassiana* was significantly higher on the cuticle of first instar larvae held in soil treated with imidacloprid at 25μg ai/g soil compared to untreated soil. Conidial germination and subsequent germ tube penetration of the cephalic capsule by *M. anisopliae* occurred within 12 hr after cuticular treatment with imidacloprid at 200 ppm ai. No conidial germination occurred on larval cuticle treated with a wetting agent. Component A was the major component of the inert carrier of the formulation responsible for stimulating conidial germination, however, the active ingredient did enhance germination at 1,000 and 10,000 ppm ai.

In several contact and oral bioassays, the combination of *B. bassiana* or *M. anisopliae* with sublethal doses of imidacloprid, synergistically increased mortality and mycosis of first instar larvae of *D. abbreviatus* in and outside soil. In addition, the time to death was reduced significantly by the fungal/chemical combination. The active ingredient was the main component of the chemical formulation responsible for the synergism with entomopathogenic fungi. Temporary larval paralysis caused by the chemical resulted in loss of mobility that, in turn, prevented normal removal of conidia from the cuticle upon contact with the surface of a given substrate. Microscopic examination of the larval cuticle showed that conidia were readily detached as they crawled over the surface of a food source or within the soil. Enhanced germination of fungal conidia by the inert carrier also synergized the fungal infection process.

CHAPTER 1
INTRODUCTION

The broad-nose weevil, *Diaprepes abbreviatus*, was introduced into Florida from Puerto Rico on nursery plants in 1964 (Woodruff 1964, 1985) and has spread to 19 counties throughout the state during the past 30 years (McCoy and Simpson 1994). Attempts to eradicate the pest during the 1970s were unsuccessful, probably due to its broad host range. Currently, an estimated 152,960 acres are infested with this weevil. Much of the infested commercial citrus area is exhibiting severe decline or is out of production. Annual crop loss is estimated at 75-100 million dollars within the Caribbean region (Hall 1995).

The biology of *D. abbreviatus* is typical of many curculionid weevils (Futch and McCoy 1994). The adults emerge from the soil, feed and oviposit on a preferred host plant. As the eggs hatch from masses laid between the leaves, larvae fall to the soil surface and burrow into the soil to feed on the roots of plants. After 8 to 15 months, the larvae pupate, adults emerge after a few weeks, and subsequently leave the soil to begin again. The duration of the cycle ranges from 8 to 24 months. Adults feed on leaves of citrus and other plant hosts, whereas the larvae also appear to be polyphagous with a particular affinity to bore into the plant roots. Larval injury to citrus is characterized by the destruction of fibrous roots and the cortical layer of both lateral and crown roots.

For many years, chlorinated hydrocarbons were applied as a barrier to the soil

beneath the tree canopy for control of first instar larvae (Bullock 1985). Following their cancellation, however, organophosphates and carbamates were used resulting in lesser control. Because of environmental risks, insecticidal usage declined gradually during the past decade (McCoy and Simpson 1994). Presently, no chemical pesticides are used for larval control in the field, but considerable research is underway to find new biological and chemical controls.

All developmental stages of *D. abbreviatus* and the other species of root weevils found in Florida are attacked by numerous natural enemies (McCoy and Simpson 1994). Various species of entomopathogenic fungi, including *Metarhizium anisopliae* and *Beauveria bassiana*, infect both larval and adult stages of citrus root weevils (Beavers et al. 1972, 1983).

A multi-phasic IPM strategy is being developed for control of larval citrus root weevils that includes soil application of entomopathogenic nematodes and fungi for control of neonatal larvae at the soil surface before the larvae reach the plant roots (McCoy and Simpson 1994). The soil ecosystem beneath the citrus tree canopy is generally shaded thereby moderating soil temperatures, reducing solar radiation, and minimizing loss in soil moisture. Although these factors appear favorable for fungal survival and their potential use as microbial control agents, field results using commercially-produced conidia of *B. bassiana* and mycelia of *M. anisopliae* showed that root weevil larval populations are suppressed in groves only when applied at extremely high inoculum rates (McCoy 1991, Schwarz 1995), suggesting that soil insect larvae are somewhat resistant to these diseases and/or the soil is strongly fungistatic (Wraight and Roberts 1987, Keller and Zimmermann 1989, McCoy and

Quintela 1994). Also, fungal persistence in the soil has usually been short (Storey et al. 1989, Gaugler et al. 1989, Studdert at al. 1990, Li and Holdom 1993, Quintela et al. 1992, 1994).

Many environmental factors impact either directly or indirectly on both persistence and survival of various fungi in nature (Roberts and Campbell 1977, Fuxa 1987, McCoy et al. 1988, 1990). An estimated 464 species of fungi, numerous bacteria, filamentous yeasts, and actinomycetes are found in soils (Kendrick and Parkinson 1990). These naturally occurring microbes and their secondary metabolites appear to function as antagonists under certain soil conditions, influencing both infectivity and survival of entomopathogenic fungi (Lingg and Donaldson 1981, Shields et al. 1981, Fargues et al. 1983, Fargues and Robert 1985, Quintela et al. 1992).

The optimal soil temperature for fungal persistence and survival varies widely according to fungal strain, soil type, soil moisture, and natural antagonists. Conidial persistence of *B. bassiana* and *M. anisopliae* appear to increase with a decrease in temperature and also decrease as soil moisture approaches saturation (Lingg and Donaldson 1981, Studdert et al. 1990, Quintela et al. 1992). Persistence declines rapidly at temperatures greater than 30^0C and 50^0C is lethal.

Soil moisture also appears to influence mycosis. Krueger el al. (1991) and Studdert and Kaya (1990), demonstrated that a proportionately greater percentage of chinch bugs, *Blissus leucopterus leucopterus* and the soybean caterpillar, *Spodoptera exigua* became infected by *B. bassiana* when exposed to drier soils compared to wetter soils.

If the source of infection remains only on the surface of the soil, only insects moving

on or coming in contact with the surface will be exposed to the microbe. Research by Storey and Gardner (1988) demonstrated that the majority (>94%) of *B. bassiana* conidia applied to the surface of light clay soil remained in the upper 5 cm of the four soil profiles tested. Only small percentages of conidia penetrated to the lower depths sampled. However, conidia readily move within sandy citrus soil, particularly after saturation with free water (McCoy unpublished data).

Frequently, inconsistent control by fungi in the field is the result of a poor understanding of the relationship between fungus, insect population, and the soil environment (Villani et al. 1992). Studies have demonstrated that soil-inhabiting insects have evolved defense mechanisms against infectious diseases. For example, Villani et al. (1994) demonstrated that soil application of mycelial particles of *M. anisopliae* affected the behavior of both larval and adult japanese beetle, *Popillia japonica.* Japanese beetle grubs avoided mycelial-treated soil while conidia caused no abnormal grub behavior. The grooming and tunneling activities of the termite, *Reticulitermes flavipes*, seems to play a vital defensive role against *B. bassiana* in the soil (Boucias et al. 1996). Tunneling activity by termites inhibited saprophytic growth of *B. bassiana* and grooming each other resulted in removal of infectious conidia from infested individuals, protecting them from fungal infection.

The concept of using a chemical to enhance the efficacy of entomopathogenic fungi has been tested using organophosphates, carbamates, and organochlorines such as DDT (Steinhaus 1956, Telenga 1968, Goral and Lappa 1974, Fargues 1973, 1975, Anderson et al. 1989, Hassan and Charnley 1989). These studies were, at most, exploratory and in many

respects inconsistent.

In 1991, Bayer AG introduced new pesticide chemistry with the neurotoxic chloronicotinyl insecticide, imidacloprid (Elbert et al. 1991). This nitromethylene heterocycle analogue is systemic in plants and is effective against a wide range of insects, including citrus root weevils (Abbink 1991, Elbert et al. 1991, McCoy et al. 1995). Imidacloprid has a neurotoxic mode of action in insects, with low mammalian toxicity (Elbert et al. 1991; Liu and Casida 1993), and degrades rapidly in the soil (Schroeder and Flattum 1984). Zeck (1992), while working with imidacloprid as a control for termites, fortuitously discovered that sublethal concentrations of imidacloprid applied to natural soil favoured rapid kill of termites in treated natural soil containing *M. anisopliae* and *Conidiobolus coronatus*. Strong synergism was detected when sublethal dosages of imidacloprid were combined with conidia of *M. anisopliae, Paecilomyces farinosus, C. coronatus, B. bassiana,* and *Actinomucor* sp. against the subterranean termite, *Reticulitermes flavipes* (Zeck and Monke 1992).

Since imidacloprid is a potential synergist of entomopathogenic fungi, studies were initiated to determine if this neurotoxin could synergize with *M. anisopliae* and *B. bassiana,* thereby improving control of first instar larvae of *D. abbreviatus.* Specific research objectives were as follows:

1. Determine the effect of different doses of imidacloprid on first instar larval mortality, ecdysis, and mobility of *D. abbreviatus* using contact and oral exposure with and without a soil substrate.

2. Determine the effect of imidacloprid on conidial attachment of *B. bassiana* and *M.*

anisopliae on the cuticle of first instar larvae of *D. abbreviatus* in and outside soil.

3. Determine the effect of imidacloprid on conidial germination and of *M. anisopliae* and *B. bassiana* on artificial substrates and on the cuticle of first instar larvae of D. abbreviatus.

4. Determine the effect of sublethal concentrations of imidacloprid and different concentrations of fungal conidia on mortality, mycosis, and ecdysis of first instar larvae of *D. abbreviatus* exclusive of soil.

5. Determine the effect of different concentrations and formulations of *M. anisopliae* and *B. bassiana* with and without sublethal doses of imidacloprid on the mobility, mortality, and mycosis of first instar larvae of *D. abbreviatus* at different soil moistures.

According to design, the following chapters each correspond to a specific objective of the overall research plan. The final discussion and conclusions represent an overview of the total research project.

CHAPTER 2
EFFECTS OF IMIDACLOPRID ON DEVELOPMENT, MOBILITY, AND SURVIVAL OF FIRST INSTAR LARVAE OF *Diaprepes abbreviatus* (COLEOPTERA: CURCULIONIDAE).

Introduction

A series of experiments were conducted to determine the effect of different doses of imidacloprid on first instar larval mortality, larval ecdysis, and mobility using contact and oral exposure with and without a soil substrate, as a precursor to further studies using entomopathogenic fungi with imidacloprid.

Materials and Methods

General Procedures

First instar larvae of *Diaprepes abbreviatus,* less than 48 h old, were used in all experiments. Larvae were obtained from eggs laid by field-collected adult females confined to screened cages in the greenhouse at 27 ±2°C as described by McCoy et al. (1995). Prior to each experiment, the most vigorous larvae were selected for experimentation by placing them in a plastic column, 14 cm in length and 3.0 cm in dia., through a 3 mm dia. hole at one end, using a small funnel. This device was enclosed in black paper to prevent light from entering the column. At the opposite end of the column, a light was positioned to attract the larvae through the column. As the larvae moved toward the light, they fell through an opening made in the middle of the column into a 50-ml beaker, used to support the column. Vigorous larvae were defined as those traveling 7 cm in 30 min.

In all bioassays, larvae were held at 28°C in the dark in 30 ml clear plastic cups containing a 1.5 cm dia. filter paper disc moistened in sterile distilled water and a thin slice of raw carrot as a food source. The filter paper was moistened every other day and the carrots replaced when they were desiccated or began to deteriorate.

A 21.4% formulation of Admire 2F, technical grade imidacloprid 1-[(6-chloro-3-pyridylmethyl)]-N-nitro-2-imidazolidin-imine was supplied by Bayer Corporation, Kansas City, MO, for use in all laboratory experiments.

The soil used in the bioassays was classified as a Candler fine sand (Typic quartzipsamments, hyperthermic, uncoated) with a particle size distribution of 96.7% sand, 0.8% silt, and 2.5% clay. The organic matter content was 0.80%, bulk density 1.47 g/cm^3, and pH 5.7.

Regressions analysis for the bioassays were performed on proportional data transformed to arcsine $\sqrt{x}$ using the general linear models procedure unless stated otherwise (SAS Institute 1985).

Larval Survival and Development Following Contact and Oral Exposure to Imidacloprid.

Five separate bioassays were conducted to determine the effect of exposure method and dose of imidacloprid on larval survival and development. In test 1, formulated imidacloprid diluted in sterile distilled water (SDW) to concentrations of 0, 12.5, 25, 50, and 100 ppm active ingredient (ai) was tested using three exposure methods. Cohorts of five first instar larvae were dipped for five sec. in the chemical solution, and then placed on filter paper to dry. Larvae were treated orally by feeding them throughout the experiment on small pieces of treated carrot, previously dipped in chemical for 5 min. and dried on filter paper.

The third treatment was a combination of the above exposure methods. Each treatment was replicated five times. Live and dead larvae were recorded daily for 10 d by microscopic examination at 10-16x magnification. Head capsule width of living larvae was measured microscopically at 50x magnification at the termination of the test.

In test 2, the experimental protocol was the same as for test 1 except an artificial diet was substituted for carrot and experimental results recorded at 23 d post-treatment. The artificial diet was prepared according to the procedures reported in McCoy et al. (1985). About 0.25 ml of each concentration of imidacloprid was pipetted onto the surface of the diet. In test 3, the experimental protocol was the same as for test 1, except imidacloprid was applied at 0, 100, 200, 400, and 600 ppm ai and experimental results recorded at 2 day intervals for 22 days.

In test 4, the experimental protocol was similar to test 1. First instar larvae were treated by placing approximately 70 individuals in 1.5 ml micro-centrifuge tubes and gently agitating for 30 sec. in imidacloprid solutions of 0, 50, 100, 250, 500, and 1000 ppm ai. Larvae were fed imidacloprid throughout the experiment on small pieces of carrots that were immersed in chemical for 30 min. Each treatment was replicated five times with 10 larvae per replication. Live and dead larvae as well as larval ecdysis were recorded via microscopic examination every other day for 9 d. In test 5, imidacloprid at 0, 20, 100, 200, 1,000, and 2,000 ppm ai was tested by immersing first instar larvae in the chemical solution for 30 sec. Each treatment was replicated five times with 10 larvae per replication.

Larval Mobility Following Contact Exposure to Imidacloprid.

Cohorts of 60 larvae were treated with imidacloprid at 0, 50, 100, 150, and 200 ppm ai by submersion for 30 seconds in 1.5 ml micro-centrifuge tubes. Twenty larvae were placed in the plastic column described previously. The time required for the first 10 larvae to move 7.0 cm was determined for four replicates during a 1-h period. This procedure was conducted at 0, 24, and 48 h after chemical treatment. Treatment means for larval movement in time were analyzed with the SAS general linear models procedure and compared using Tukey's Honestly Significant Difference (HSD) test at P=0.05 (SAS Institute 1985).

Larval Survival, Development, and Recovery Following Oral Exposure to Imidacloprid.

Pieces of 8 mm dia. carrot were dipped in imidacloprid solutions of 0, 50, 100, 500, and 1,000 ppm ai for 30 min. Larvae were fed treated carrot for 3 d. Carrots were then replaced with fresh untreated carrot. Larval mortality, ecdysis, and recovery were observed every other day for 9 d using a stereoscopic microscope at 10-16X magnification. Complete larval recovery from imidacloprid treatment was defined by no sluggishness or uncoordinated quivering. In addition, larval feeding was confirmed by the presence of carrot in the larval digestive tract.

Larval Mobility and Survival Following Treatment with Imidacloprid Applied to Natural and Sterilized Candler Soil.

Sandy soil was air dried at 40°C for 12 h and then sieved to remove particulate matter. A portion of soil was then sterilized by autoclaving for 4 h and oven dried for 24 h. Approximately 80 g of natural and sterilized soil moistened to 12% was added to 20 bioassay columns to a depth of 3 cm. The columns were constructed from polystyrene tubes (10.5

cm ht., 5.0 cm diam.) and except for size, were similar to bioassay columns described by Hamlen and Beavers (1975). Imidacloprid solutions at 0, 12.5, 25.0, 50.0, 100.0 ppm ai were pipetted uniformly onto the soil in bioassay columns in 4-ml aliquots. These doses were equivalent to 0, 0.625, 1.25, 2.5, and 5.0 μg/g of soil, respectively. Each treatment was replicated four times. Ten neonates were scattered on the surface of the soil, and the open bioassay units were held at 25°C. After 48 h, the number of live and dead larvae recovered in the containment cell at the base of the bioassay column was counted microscopically.

The experiment was repeated using higher doses of imidacloprid at 0, 0.50, 10.0, 20.0, and 30.0 ai μg/g soil. All live larvae recovered from the containment cell were placed in a plastic vial containing a moisten filter paper and a piece of sliced carrot as a food source and held at 28 °C. Larval mortality was recorded every other day for 17 days.

Another experiment was conducted to determine the effect of soil moisture on mobility and survival of first instar larvae in treated and untreated soil. Two milliliters of imidacloprid at concentrations of 0, 100, 500, and 1000 ppm ai were added to 30 g of sifted natural Candler soil. These doses were equivalent to 0, 6, 30, 60 μg/g of soil. The treated soil was air-dried in a dark incubator at 28°C for 18 h and then moistened with SDW to 2, 6, and 12% moisture. Two grams of treated soil were added to a depth of 1 cm to bioassay columns (15 cm ht, 2.0 cm dia.). Ten first instar larvae were placed on the soil surface of each container. Bioassay units were closed tightly and held at 28°C. Each treatment was replicated four times for each moisture. After 8 d, the number of live and dead insects recovered from the containment cell and the soil was recorded. In addition, the level of soil moisture per unit was monitored daily by weighting the soil in four replications. No water

loss was observed after 8 d.

Larval Mortality and Root Protection to Citrus Seedlings Following a Soil Drench Treatment with Imidacloprid in the Greenhouse.

Individual Swingle citrumelo rootstock seedlings, about 25 cm in length, were transplanted into 16-cm-diam. plastic pots containing Candler soil. All plants were maintained in an air-conditioned greenhouse at 27-28°C for 3 wk after transplanting to establish normal root growth. Plants were watered weekly at 300 ml/container. Imidacloprid at rates of 0, 50, 100, and 200 ppm ai was prepared in 150 ml of SDW and applied to the soil surface as a drench to each of the ten containers. Twenty plants each served as controls, 10 with and 10 without larvae. At 1 d post-treatment, 30 first instar larvae were added to the surface of the soil within each container. After 6 wk post-inoculation, each plant was removed carefully from each pot and the soil processed to recover surviving larvae. Generally, control larvae had molted 3-4 times after 4 wk on the plant roots and therefore were readily visible. In addition, gross root symptoms were determined to estimate larval injury to the plant. Fibrous roots were removed from each plant to determine dry root weight. Differences between treatments means were determine using ANOVA and Tukey's Honestly Significant Difference (HSD) (SAS Institute, 1985).

Results

Larval Survival and Development Following Contact and Oral Exposure to Imidacloprid.

In test 1, when imidacloprid was applied as a contact treatment, there was no significant difference in larval mortality after 10 d at all doses ($F=0.74$; $df=1,23$; $P<0.3983$) (Fig. 2.1-A). When imidacloprid was fed to first instar larvae on treated carrots, mean larval

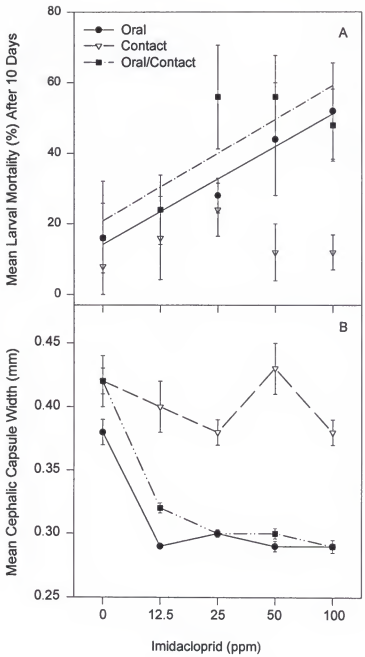

Fig. 2.1. Effect of contact, oral, and oral/contact exposure of first instar larvae of *Diaprepes abbreviatus* to imidacloprid on survivorship (A) and larval development (B) after 10 d.

mortality increased with an increase in chemical dose (r^2=0.29; F=6.4; df=1,23; P<0.0215). Contact/oral exposure caused a similar increase in larval mortality, however, mortality was highest at 25 and 50 ppm in the combination (r^2= 0.31; F=9.6; df=1,18; P<0.0053). Because of within treatment variability among replicates, the coefficients of correlation (r^2) for larval mortality and chemical dose was very low for both exposure methods. The rate of larval development, determined by measuring the width of the larval cephalic capsule, showed that oral exposure with and without contact exposure resulted in reduced larval development at all concentrations of imidacloprid (Fig. 2.1-B). Treated larvae failed to molt as there was no increase in cephalic capsule width (0.30 ±0.02 mm). In contrast, larval development and molting appeared normal for all concentrations of imidacloprid applied by contact.

In test 2, where artificial diet was substituted for carrot as a food source, similar results were obtained using the same range of doses of imidacloprid (Fig. 2.2). Again, contact exposure had no effect on larval mortality after 23 d (F=0.29; df=1,23; P<0.5940). Larval mortality increase linearly with an increase in doses of imidacloprid for both oral (r^2=0.34; F=10.91; df=1,22; P<0.0032) and oral/contact exposure (r =0.48; F=18.88; df=1,22; P<0.0003) (Fig. 2.2-A). As was true in the previous test, larvae fed treated diet failed to molt while larvae dipped in different concentrations of imidacloprid completed normal larval ecdysis (Fig. 2.2-B).

In test 3, the effect of oral and contact exposure to imidacloprid at higher doses ranging from 0-600 ppm is shown in Fig. 2.3. At doses of 400 ppm or less, there was no effect in larval mortality after 22 days following contact exposure, however, at 600 ppm mortality reached 57.7% (Fig. 2.3-A). The regression analysis was significant only for the

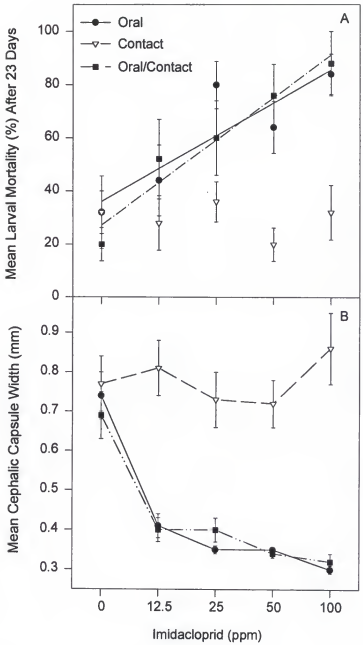

Fig. 2.2. Effect of contact, oral, and oral/contact exposure of first instar larvae of *Diaprepes abbreviatus* to imidacloprid on survivorship (A) and larval development (B) on an artificial medium after 23 d.

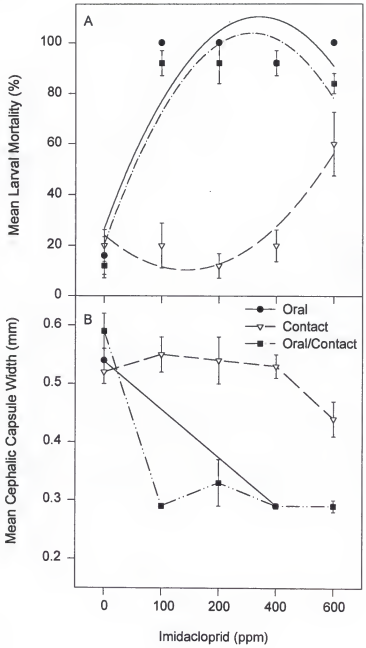

Fig. 2.3. Effect of oral, contact, and oral/contact application of imidacloprid to first instar larvae of *Diaprepes abbreviatus* on survivoship (A) and larval development (B).

quadratic curve (R^2=0.27; F=6.62; df=2,22; P<0.0174). By contrast, when imidacloprid was fed to first instar larvae on treated carrots, mean larval mortality ranged from 80-100% at all doses from 100 to 600 ppm, and the relationship fit the quadratic for both oral/contact treatment (R^2=0.78; F=11.61; df=2,22; P<0.0025) and oral exposure (R^2 =0.88; F=15.47; df=2,22; P<0.0007). In the case of larval development, oral exposure again affected ecdysis while contact exposure had no effect.

The results for test 4, where doses of imidacloprid at 50 to 1000 ppm were applied orally or as a contact treatment to first instar larvae, are presented in Fig. 2.4. Following contact application, larval mortality increased linearly with an increase in imidacloprid dose (r^2=0.37; F=17.41; df= 1,28; P<0.0003) (Fig. 2.4-B). Even at 1000 ppm, only 50% of larvae were killed after 9 days. When fed carrot treated with imidacloprid, however, percent larval mortality in time also increased with an increase in the dose, fitting the quadratic equation (R^2=0.65; F=13.82; df= 2,27; P<0.0009) (Fig. 2.4-A). By comparison, percent larval mortality following oral exposure was higher at all chemical doses greater than 50 ppm.

All concentrations of imidacloprid ranging from 50-1000 ppm applied orally or to the larval cuticle influenced larval ecdysis (Fig. 2.4-A, B), in fact, larvae fed imidacloprid on treated carrot failed to molt (R^2=0.37; F=8.76; df= 2,27; P<0.0063). All doses affected larval behavior within a few hours after feeding. Many larvae stopped feeding and some exhibited abnormal locomotory behavior such as tremors and tumbling. When imidacloprid was applied to the larval cuticle however, ecdysis decreased with an increase in doses up to 500 ppm and the relationship appeared to best fit the quadratic model (r^2=0.68; F=13.72; df=2,27; P<0.001).

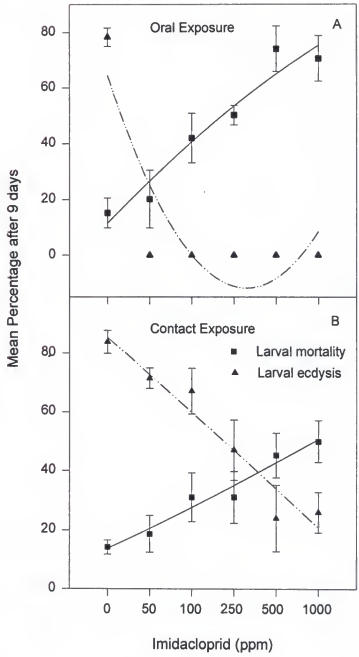

Fig. 2.4. Effect of oral (A) and contact application (B) of imidacloprid to first instar larvae of *Diaprepes abbreviatus* on mortality and ecdysis after 9 d.

In test 5, first instar larval mortality gradually increased with an increase in dose following contact exposure using the immersion method (Fig. 2.5). The relationship between chemical dose and larval mortality increased after 3 (R^2=0.48; F=8.03; df=2,27; P<0.0086), 6 (R^2=0.71; F=25.22; df=2,27; P<0.0001), and 9 days post-treatment (R^2 =0.74; F=26.04; df=2,27; P<0.0001) and best fit a quadratic curve particularly after 6 and 9 days when mortality was highest. At concentrations of 100 ppm and higher, larvae appeared lethargic and ecdysis significantly declined (R^2=0.79; F=31.88; df=2,27; P<0.0001) and the relationship between chemical dose and ecdysis best fit a quadratic curve. At concentrations of 1,000 ppm and 2,000 ppm, larvae molting declined to less than 10%.

Larval Mobility Following Contact Exposure to Imidacloprid.

The mobility of first instar larvae declined in time with an increase in the concentration of imidacloprid following contact treatment (Fig. 2.6). A negative quadratic relationship between mobility and chemical dose was found at 0 h and changed to a linear relationship at 24, and 48 h post-treatment. Immediately following treatment (0 h), larval mobility decreased significantly at 25, 50 and 100 ppm (R^2=0.88; F=14.55; df=2,21; P<0.001) and larvae were totally immobile at 150 and 200 ppm. At 24 and 48h post-treatment, larval mobility was significantly different among chemical doses (r^2=0.62; F=35.48; df=1,22; P<0.0001), (r^2=0.71; F=47.89, df=1,22; P<0.0001), but mobility increased with time at all doses, suggesting some loss in chemical effect. In fact, at 24 and 48 h post-treatment, 100% of the larvae exhibited normal movement at doses of 25 and 50 ppm.

Larval mobility measured in time was also affected by doses (Fig. 2.6). At time zero, larvae treated with imidacloprid at 100 ppm or less required 8-12 min. to travel 7 cm

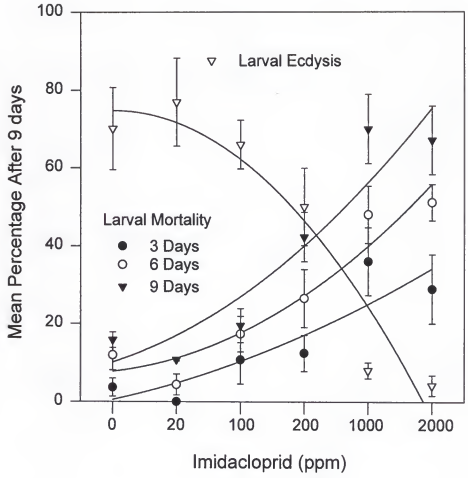

Fig. 2.5. Effect of imidacloprid applied to the cuticle on survival and ecdysis of first instar larvae of *Diaprepes abbreviatus*.

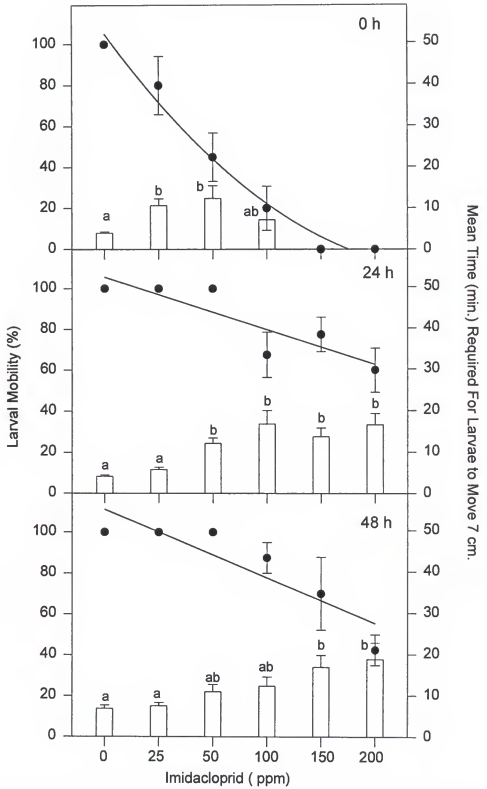

Fig. 2.6. Mean percent larval mobility (dots), and mean time required for first instar larvae of *Diaprepes abbreviatus* to move 7 cm (bars), at 0, 24, and 48 h after cuticular treatment with imidacloprid. Bars with the same letter are not significantly different by Tukey's test (P=0.05).

compared to 4 min for the control larvae. At higher doses (150-200 ppm) where larvae were immobile at 0 h, they now required 15-18 min to travel the defined distance. Generally, larval mobility in time was significantly affected at doses of imidacloprid at 50 ppm and higher.

Larval Survival, Development, and Recovery Following Oral Exposure of Imidacloprid.

Some first instar larvae fed treated carrot for 3 d regained normal activities and even completed molting when fed untreated carrot (Fig. 2.7). A similar negative quadratic relationship was observed between larval ecdysis and chemical doses (R^2=0.86; F=10.33; df=2,22; P<0.004) and larval recovery and chemical doses (R^2=0.86; F=10.09; df=2,22; P<0.0044). These results suggest that larval ecdysis is a good indicator of larval recovery from paralysis from imidacloprid treatment. Larval mortality increased linearly with the dose of imidacloprid (r^2=0.60; F=33.96; df=1,23; P<0.0001). However, percent larval mortality was less than for test 4 where larvae were fed treated carrots continuously at concentrations higher than 500 ppm (Fig. 2.4-A). After replacement of the treated carrots, larval ecdysis was 56 and 26% at 50 and 100 ppm, respectively (Fig. 2.7) compared with no ecdysis even at 12.5 ppm when larvae were exposed continually to treated carrot (Figs. 2.1, 2.2, 2.3).

Larvae Mobility and Survival Following Treatment with Imidacloprid to Natural and Sterilized Candler Soil.

In test 1, the vertical movement of first instar larvae through soil at 12% moisture decreased with an increase in the dose of imidacloprid in both autoclaved (R^2=0.72; F=17.47; df=2,17; P<0.0006) and natural soil (R^2=0.53; F=7.38; df=2,17; P<0.0146) and best fit

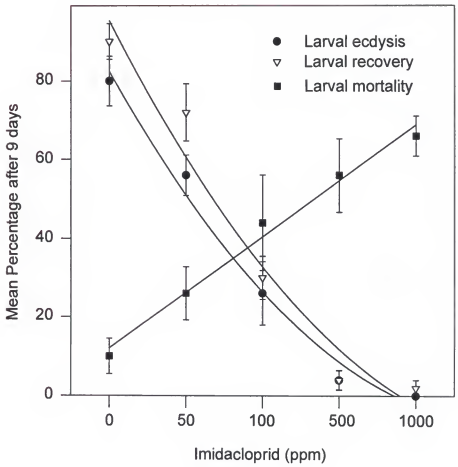

Fig. 2.7. Effect of oral exposure of first instar larvae of *Diaprepes abbreviatus* on larval mortality, ecdysis, and recovery. Complete larval recovery from imidacloprid treatment was defined by no sluggishness or uncoordinated quivering.

quadratic curves in both cases (Fig. 2.8). Overall larval movement was lowest in autoclaved soil. When autoclaving and oven drying Candler sandy it became eletrostactic and probably affected larval movement. At 5μg/g soil, only 12.5 and 50% of the larvae moved through both autoclaved and natural soil, respectively.

In test 2, larval movement in sterilized soil decreased linearly with an increase in the dose of imidacloprid (r^2=0.70; F=38.52; df=1,18; P<0.0001) (Fig. 2.9-A). In natural soil, a negative quadratic regression was observed between larval movement and doses of imidacloprid (r^2=0.71; F=19.64; df=2,17; P<0.0004). After 17 days, larval mortality with chemical treatments for natural (r^2=0.19; F=3.78; df=1,18; P<0.0686) and autoclaved soil (r^2=0.17; F=0.04; df=1,11; P<0.8487) were similar to that in the control (Fig. 2.9-B). These results suggest that the larvae after passage through chemically treated soil recovered and assume normal behavior.

In test 3, larval mobility in natural Candler soil was not affected by moisture level in the controls (Fig. 2.10-A). However, larval mobility decreased significantly with an increase in dose of imidacloprid at all moistures (Table 2.1). Relationship between mobility and chemical dose and moisture best fit a quadratic and a linear equation, respectively (Table 2.1). Larval mobility in treated soil appeared to decrease as soil moisture increased, for example, at 100 ppm only 22.4% of the larvae were recovered from soil at 12% moisture compared to 67 and 70% recovery at 6 and 2% moisture, respectively.

Larval mortality among those remaining in the soil after 8 d was not significantly affected by soil moisture (Fig. 2.10-B, Table 2.1). In treated soil, mortality varied significantly for the different doses of imidacloprid, in fact, a dose response was found only

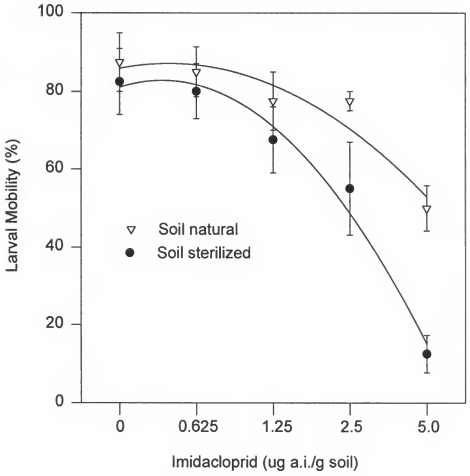

Fig. 2.8. Effect of imidacloprid on mobility of first instar larvae of *Diaprepes abbreviatus* in sterilized and natural soil.

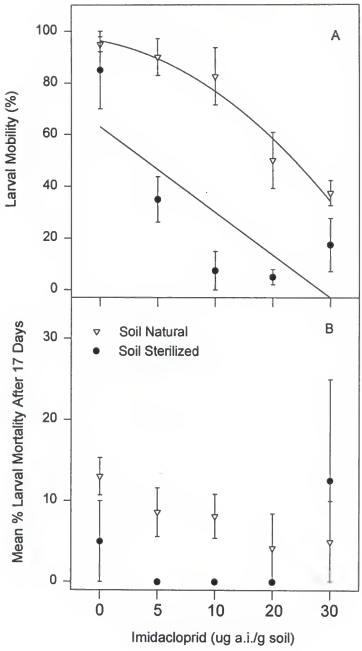

Fig. 2.9. Effect of imidacloprid on mobility (A) and survival (B) of first instar larvae of *Diaprepes abbreviatus* in sterilized and natural soil.

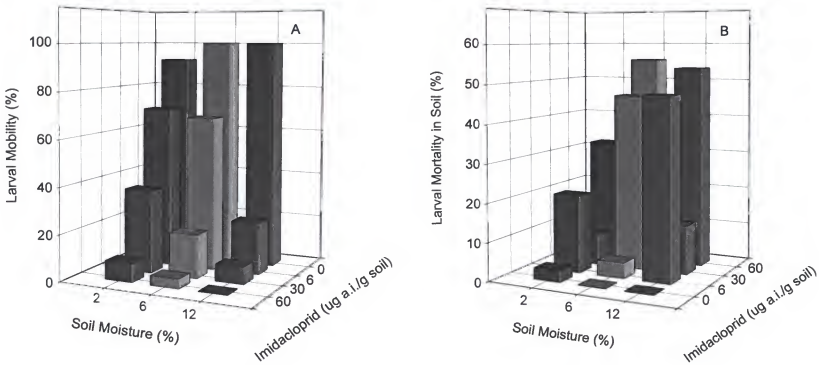

Fig. 2.10. Effect of imidacloprid on mobility (A) and survival (B) of first instar larvae of *Diaprepes abbreviatus* in natural candler soil at different moistures.

Table 2.1. Summary of multiple regression analysis for larval mobility and mortality of *Diaprepes abbreviatus* subsequent to soil treatment with imidacloprid (IMI) under different soil moistures (Moist).

Factor	Larval Mobility (R^2=0.81)[a]			Larval mortality (R^2=0.35)[a]		
	F	df	P value	F	df	P value
IMI	171.44	1,53	0.0001	20.95	1,53	0.0001
IMIxIMI	24.64	2,52	0.0001	0.0	2,52	0.9575
Moist	7.67	1,53	0.0078	1.99	1,53	0.1644
MoistxMoist	0.59	2,52	0.4440	0.40	2,52	0.5294
IMIxMoist	0.08	2,52	0.7784	0.01	2,52	0.9274

[a] R-square of the model on data tranformed to arcsine √proportion.

at 6% soil moisture.

Larval Mortality and Root Protection to Citrus Seedlings Following a Soil Drench with Imidacloprid in the Greenhouse.

Imidacloprid at rates of 50, 100, and 200 ppm significantly reduced larval populations compared to the control (Table 2.2). The mean fibrous root mass was significantly lower in all chemical treatments compared to untreated control without larvae. However, fibrous root mass was significantly greater at 200 ppm compared to lower rates. At 100 ppm and 200 ppm, there was no detectable root injury to the plants, suggesting feeding inhibition and/or systemic activity at these rates.

Table 2.2. Effect of imidacloprid applied as a soil drench on the control of first instar larvae of *Diaprepes abbreviatus* in container-grown citrus in the greenhouse.

Treatment	Rate (ppm)	Mean[a] no. live larvae/ plant ± SEM	Mean[a] fibrous root mass (mg dry wt ± SEM)	No. of girdled plants	Gross root symptomology
Control (no larvae)	0	0.0 b	13.1 ± 1.0 a	0	Healthy plants
Control (larvae)	0	7.9 ± 1.42 a	6.2 ± 0.03 c	10	Severe stem girdling and bark injury
Imidacloprid	50	2.2 ± 1.23 b	8.1 ± 0.43 bc	3	Partial stem girdling and bark injury
Imidacloprid	100	0.4 ± 0.4 b	8.2 ± 0.25 bc	0	Healthy plants
Imidacloprid	200	0.0 b	8.6 ± 0.21 b	0	Healthy plants

[a] Means based on 10 replications per treatment. Means followed by the same letter are not significantly different at the 5% level of probability using Tukey's HSD test.

CHAPTER 3
EFFECT OF IMIDACLOPRID ON CONIDIAL ATTACHMENT OF *Metarhizium anisopliae* AND *Beauveria bassiana* TO THE *Diaprepes abbreviatus* (COLEOPTERA: CURCULIONIDAE) LARVAL CUTICLE.

Introduction

The attachment of conidia of the entomopathogenic fungi to the host cuticle represents the initial event in the infection process (Boucias et al. 1988). Fargues (1984) proposed that adhesion of conidia to the cuticle involves two stages, an adsorption and a consolidation stage. Adsorption of conidia to the cuticle is a non-specific process mediated by electrostatic and hydrophobic events (Boucias et al. 1988, Boucias and Pendland 1991). Conidia of many deuteromycetes have a dry hydrophobic surface and exhibits an outer layer comprised of interwoven fascicles of rodlets and possesses a net negative charge (Boucias et al.1988, Boucias and Pendland 1991). At the consolidation stage, chemical cues associated with the host cuticle passively stimulate adsorbed pregerminated conidia to become "actively" attached to the cuticle by inducing either certain enzymatic processes and/or secretion of mucilagenous substances (Fargues 1984).

Both the topography and chemistry of the cuticle can affect conidial adhesion of entomopathogenic fungi (Sosa-Gomez et al. 1996). Proteins from the cuticle of the exuvia of the southern stink bug, *Nezara viridula*, did not affect conidial adhesion, but solvent extraction of cuticular lipids significantly reduced adhesion of *M. anisopliae* conidia (Sosa-Gomez et al. 1996). In several studies, *M. anisopliae* conidia have shown an affinity to those

regions containing setae or spines (Boucias et al.1988, Sosa-Gomez et al. 1996) and to highly hydrophobic cuticle regions, such as the mosquito siphon tube (Lacey et al. 1988).

In this chapter, conidial attachment of *M. anisopliae* to different regions on imidacloprid treated and untreated cuticle was examined using first instar larvae of *D. abbreviatus*. The effect of dose of imidacloprid and each component of the formulation separately on conidial attachment were determined on larval cuticle. In addition, the effect of imidacloprid on removal and attachment of *M. anisopliae* conidia to the larval cuticle was determined at different soil moistures.

Materials and Methods

General Procedures.

All procedures relating to insect larvae, chemical, and soil were the same as described in chapter 2.

M. anisopliae isolated from *D. abbreviatus* larvae collected from soil at Apopka, FL and designated MADA strain and *B. bassiana* isolated from a grasshopper and commercialized as Mycotrol WP by Mycotech Corporation, Butte, MT were used in the experiments. Conidia of both fungi were harvested from fungal cultures produced on potato dextrose agar plus 0.4% yeast extract (PDAy) for 10-15 days at 25±1°C. Original conidial inoculum was taken from pure cultures, no more than two serial passages from a host insect. Conidial viability was determined by counting germ tubes produced on PDAy after 18 h using a phase contrast microscope at 400x. Conidial viability was always 98-100%.

Attachment of Conidia of *Metarhizium anisopliae* to Different Regions of Larval Cuticle Treated with Imidacloprid in Time.

Conidia of *M. anisopliae* and *B. bassiana* were suspended in 0.05 M carbonate - bicarbonate buffer, pH 9.2 in Tween 80 0.01%. The suspensions were vortexed at high speed and then filtered through a cloth screen and sequentially washed in distilled sterilized water by centrifugation at 1,000 relative centrifugal force (RCF) for 5 min. The conidia were stained with 1 mg of fluorescein isothiocyanate (FITC, Sigma Chem.) in 1 ml of carbonate-bicarbonate buffer for 1 hr at room temperature according to Hung and Boucias (1992). Labeled conidia were washed five times with sterile distilled water prior to being used in the experiments.

Approximately 50 first instar larvae were placed in 1.5 ml micro-centrifuge tubes containing FITC-labeled conidia of *M. anisopliae* at a concentration of 10^7 conidia/ml or in a tube containing the same number of conidia and imidacloprid at 100 ppm. The tubes were agitated gently for 30 seconds before larval removal. Larvae were held individually in tissue culture plates (well size 2.0 mm dia.) at 28 °C in the dark. Each well contained an 8-mm dia. slice of raw carrot as a food source. At 0, 12, and 24 h post-treatment, larvae were transferred to a freezer at -50°C for 30 min. Dead larvae were mounted in 1,4-diazabicyclo[2.2.2]octane. Conidial attachment on the cuticle was examined using an epifluorescent Leica microscope with an exciter filter BP 450-490 nm and a barrier filter of 0-530 nm at 600x in an area of cuticle of 0.013 mm^2 Twenty five fields were examined at 0 h, and 50 fields at 12 and 24 h for each treatment. Photographs were taken using a TMAx 400 ASA 35-mm film.

A preliminary test was conducted to determine the most efficient preparatory method to use to determine conidia number present on carrot. Carrots (8 mm-dia.) were submerged for 5 min. in a suspension of *M. anisopliae* at 10^6 conidia/ml. Six carrots were held in 30 ml plastic container in 5 replications at 28 °C. After 24 h, an individual piece of carrot was vortexed at high speed for 30 seconds in 400 μl of Tween 80 0.05%. In the second method, the carrot was macerate with mortar and pestle in 400 μl of Tween 80 0.05%. Two hundred μl of each suspension were then spread in 90 mm dia. Petri dish containing dodine (0.46 g ai/ liter) oatmeal agar media (Beilharz et al. 1982). Plates were incubated at 28°C After 7 days, the number of colony forming units (CFU's) were counted in 30 dishes for each method. By macerating the carrots conidial recovery was 7.0 times more efficient than the other method. Maceration recovered 455.6 ± 32.0 conidia of *M. anisopliae* compared to 66.2 ± 16.8 using the vortex.

The maceration method was used to detemine the presence of *M. anisopliae* conidia on the carrots used to feed larvae of *D. abbreviatus* in 40 replications or Petri dishes as described previously. The plastic wells that held larvae were also tested for the presence of *M. anisopliae* conidia. This was done by adding 200 μl of Tween 80 0.05% to each well and brushing the conidia from the container walls. A 200 μl suspension was plated onto 90 mm Petri dish containing the dodine oatmeal media and mantained at 28°C. After 7 days the number of colony forming units were counted in 37 dishes.

Conidial Attachment of *Metarhizium anisopliae* on Larval Cuticle Treated with Different Doses of Formulated Imidacloprid and Each component Separately .

In test 1, approximately 50 first instar larvae were placed in 1.5 ml micro-centrifuge tubes containing FITC-labeled conidia of *M. anisopliae* at concentration of 10^7 conidia/ml or in combination with imidacloprid at 100 ppm, 500, and 1,000 ppm ai. The tubes were agitated gently for 30 seconds. After treatment, dead larvae were mounted in 1,4-diazabicyclo[2.2.2]octane. Conidial attachment to the cuticle was examined at 630x magnification using an epifluorescent Leica microscope. One field of ten larvae was examined for each treatment. Regression analyses were performed on the data using the general linear models procedure (SAS Institute 1985).

In test 2, formulated imidacloprid at 500 ppm, active ingredient (ai) at 500 ppm, and inert carrier at 1,836 ppm (concentration equal to the amount found in formulated imidacloprid at 500 ppm of the active ingredient) were mixed with FITC labeled conidia of *M. anisopliae* at concentrations of 0, and 10^7 conidia/ml. Larvae were dipped in each suspension in the manner described previously. Conidial attachment on the cuticle was examined within a microscopic field at 630x magnification. One field of ten larvae was examined for each treatment.

Conidial Attachment of *Metarhizium anisopliae* and *Beauveria bassiana* on Insect Cuticle in Soil Treated with Imidacloprid at Different Moistures.

One-half ml of suspension containing FITC-labeled conidia of *M. anisopliae* at 10^7 and 10^8 conidia/ml or *B. bassiana* at 10^8 conidia/ml were added alone or in combination with imidacloprid at a concentration of 500 ppm to 30 g of Candler soil. These concentrations were equivalent to *M. anisopliae* at $5x10^5$ and $5x10^6$ conidia/g soil, *B. bassiana* at $5x10^6$

conidia/g soil, and imidacloprid at 25 μg a.i./g soil, respectively. Soil moisture was adjusted to 7 and 14% by adding sterile distilled water. For 0% moisture, treated soil was air-dried in a dark incubator at 28^0 C with continous air flow. For the test involving *B. bassiana,* soil was held at 7% moisture. Two g of treated soil were added to a depth of 1 cm to bioassay columns (15 cm ht, 2.0 cm diam.) constructed from polystyrene tubes similar to that described by Hamlen and Beavers (1975). Bioassay columns were attached to a containment cell. Twenty 48 hr old first instar larvae were placed on the soil surface of each container. Bioassay units were closed tightly and held at 28° C. Each treatment was replicated 3 times. After 22 h, larvae recovered from containment cells and the soil in each tube were mounted in DABCO. Conidial attachment to the larval cuticle was examined at 630 x using an epifluorescent Leica microscope. Photographs were taken using a TMAx 400 Asa 35-mm film.

Removal of *M. anisopliae* Conidia from the Larval Cuticle of *D. abbreviatus* Treated with Imidacloprid in Soil at Different Moistures.

First instar larvae were submerged in a suspension of FITC labeled conidia at 10^7 conidia/ml or in a imidacloprid conidial mixture at 0, or 500 ppm ai for 30 sec. Twenty inoculated larvae were placed on the soil surface in bioassay columns (1 cm depth). Soil moisture was adjusted to 0, 7, or 14% (v/w). Each treatment was replicated 4 times. After 24 h, the number of conidia attached to the larval cuticle was counted with a fluorescence microscope. A total of 15 microscopic fields for each treatment were examined at 630 x .

Qualitative Examination of Conidial Attachment of *M. anisopliae* on Insect Cuticle Using Scanning Electron Microscope.

Larvae treated with *M. anisopliae* at 10^7 conidia/ml were prepared for scanning

electron microscopy using a fixation process described by Grodowitz et al. (1982). The larvae were then coated with gold/palladium (80:20) and examined with a Hitachi S-530 scanning electron microscope operating at 20 kV. Photographs were taken with polaroid 55 film.

Statistical analysis.

All bioassays data were analyzed using general linear model procedure (Proc GLM) and treatment means compared using least square means (LSMEANS) unless stated otherwise (SAS Institute 1985).

Results

Attachment of Conidia of *Metarhizium anisopliae* to Different Regions of Larval Cuticle Treated with Imidacloprid in Time.

FITC-labeled conidia of *M. anisopliae* applied with and without imidacloprid were evenly attached to the cuticle of 10 abdominal segments of larvae of *D. abbreviatus* (Fig. 3.1, Tables 3.1, 3.2). No significant difference was found for conidial attachment between treated and untreated larvae ($P<0.1089$). At 12 h post-treatment, the removal of conidia from the cuticle was uniform on all abdominal segments for all treatments (Fig. 3.1, Tables 3.1, 3.2). However, untreated larvae removed significantly more conidia from the cuticle than larvae treated with the chemical ($P<0.001$).

Conidial attachment to various regions of larval cuticle is presented in Fig. 3.2. Immediately after treatment, no difference was observed on conidial attachment to the cuticle of untreated larvae compared to treated larvae ($P>0.9972$). Conidia of *M. anisopliae* tended to attach to epicuticular folds, around sensilla organelles or in depressions of the cuticle of

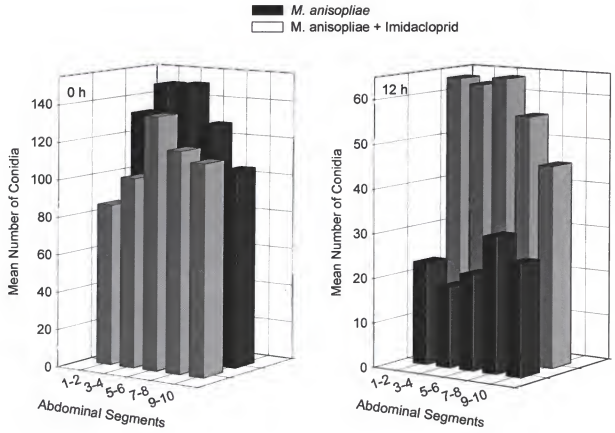

Fig. 3.1. Mean number of conidia of *Metarhizium anisopliae,* applied alone or in combination with imidacloprid at 100 ppm ai, attached to abdominal segments of the cuticle of first instar larvae of *Diaprepes abbreviatus.* Mean values of attached conidia counted within a microscope field (x600) having an area of 0.013 mm^2 (25 and 50 fields for each treatment at 0 and 12 h post-treatment, respectively).

Table 3.1. P values of least square means analysis of the mean number of conidia of *Metarhizium anisopliae* attached to abdominal segments of the cuticle of first instar larvae of *Diaprepes abbreviatus*.

Abdominal segments	1-2	3-4	5-6	7-8	9-10
0 h					
1-2	-	0.5536	0.2687	0.2751	0.4944
3-4	0.5536	-	0.5985	0.6091	0.9264
5-6	0.2687	0.5985	-	0.9877	0.6636
7-8	0.2751	0.6091	0.9877	-	0.6747
9-10	0.4944	0.9264	0.6636	0.6747	-
12 h					
1-2	-	0.7501	0.9223	0.6262	0.8862
3-4	0.7501	-	0.8250	0.4217	0.6446
5-6	0.9223	0.8250	-	0.5591	0.8099
7-8	0.6262	0.4217	0.5591	-	0.7305
9-10	0.8862	0.6446	0.8099	0.7305	-

Table 3.2. P values of least square means analysis of the mean number of conidia of *Metarhizium anisopliae* attached to abdominal segments of the cuticle of first instar larvae of *Diaprepes abbreviatus* treated with imidacloprid.

Abdominal segments	1-2	3-4	5-6	7-8	9-10
0 h					
1-2	-	0.8078	0.3188	0.6086	0.2355
3-4	0.8078	-	0.4469	0.4522	0.1572
5-6	0.3188	0.4469	-	0.1386	0.0362
7-8	0.6086	0.4522	0.1386	-	0.4902
9-10	0.2355	0.1572	0.0362	0.4902	-
12 h					
1-2	-	0.9421	0.9684	0.6161	0.2330
3-4	0.9421	-	0.9106	0.6679	0.2620
5-6	0.9684	0.9106	-	0.5886	0.2181
7-8	0.6161	0.6679	0.5886	-	0.4849
9-10	0.2330	0.2620	0.2181	0.4849	-

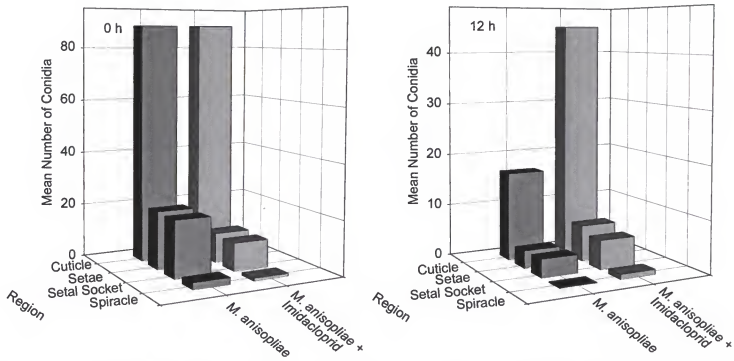

Fig. 3.2. Mean number of conidia of *Metarhizium anisopliae,* applied alone or in combination with imidacloprid at 100 ppm ai, attached to different sites of the cuticle of first instar larvae of *Diaprepes abbreviatus.* Mean values of attached conidia counted within a microscope field (x600) having an area of 0.013 mm^2 (25 and 50 fields for each treatment at 0 and 12 h post-treatment, respectively).

D. abbreviatus larvae (Fig. 3.3-A, B, C). Conidia were also attached to setae and around setal sockets (Fig. 3.4-A, B). At 0 h, conidial attachment on setae ($P<0.0002$) and around setal sockets ($P<0.0002$) was significantly higher for fungus alone compared to imidacloprid treated larvae (Fig. 3.2). Conidial attachment to the spiracular region was very low for both control and imidacloprid-treated larvae and no difference was observed between treatments ($P>0.2119$). At 12 h post-treatment, conidial attachment was significantly lower for larvae treated with fungus alone compared to the combination on the cuticle ($P<0.0001$), setae ($P<0.0083$), and spiracles($P<0.0033$) (Fig. 3.2). There was no difference in attachment of conidia to the setal socket between control and treated larvae ($P>0.2094$).

A marked reduction was observed over time in the numbers of FITC-labeled conidia associated with the cuticle of untreated larvae (Fig. 3.5). Within 12 h post-treatment, 82% of the conidia were removed from the larvae. By 24 h, only 2.2 conidia per microscope field were observed on larval cuticle. Many larvae examined contained no conidia. Conidia on larvae exposed to imidacloprid did not show this marked reduction in conidial numbers (Fig. 3.5). At 12 h post-treatment, imidacloprid-treated larvae contained 58.2 conidia per microscope field, which was significantly different from control larvae ($P<0.0001$). By 24 h, 53.5% of conidia were still present on imidacloprid-treated larvae and the number of conidia was significantly different from control ($P<0.0001$).

Conidia were readily detached from larvae treated with fungus-only as they crawled over the surface of the food source (carrot) and the walls of the plastic container. Conidial residues were significantly higher on the carrots ($P<0.0001$) and the inner wall of plastic cups ($P<0.0001$) contained control larvae compared to imidacloprid-treated larvae (Fig. 3.6).

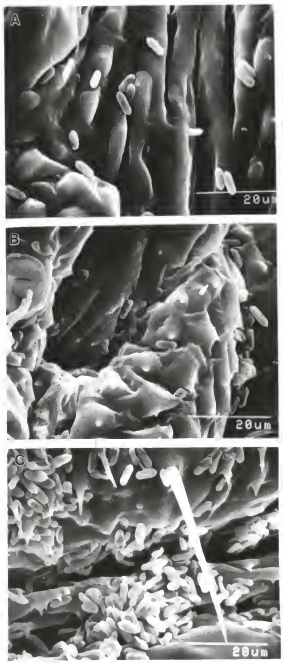

Fig. 3.3 A, B, C. Scanning electron micrographs of *Metarhizium anisopliae* conidia attached to cuticle of first instar larvae of *Diaprepes abbreviatus*.

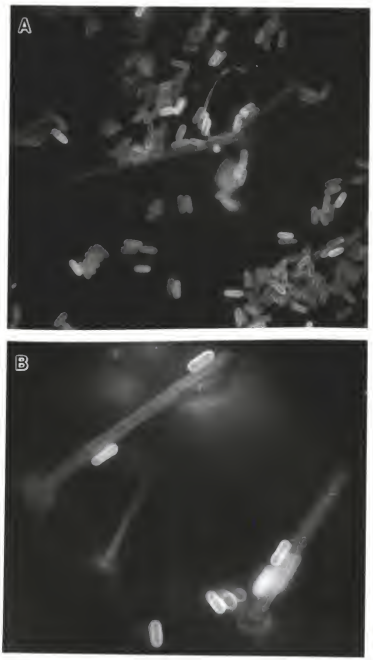

Fig. 3.4. Epifluorescence photomicrograph of FITC labeled conidia of *Metarhizium anisopliae* attached to larval cuticle (A) and setae (B) of *Diaprepes abbreviatus*.

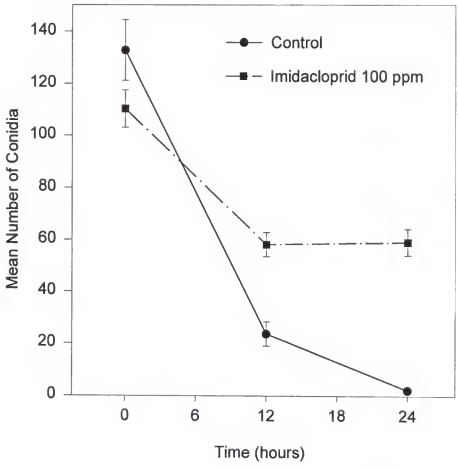

Fig. 3.5. Mean number of conidia of *Metarhizium anisopliae,* applied alone or in combination with imidacloprid at 100 ppm ai, attached to the cuticle of first instar larvae of *Diaprepes abbreviatus* at 0, 12 and 24 h post-treatment. Mean values of attached conidia counted within a microscope field (x600) having an area of 0.013 mm^2 (25 five fields were examined at 0 h and 50 fields at 12 and 24 h for each treatment, respectively).

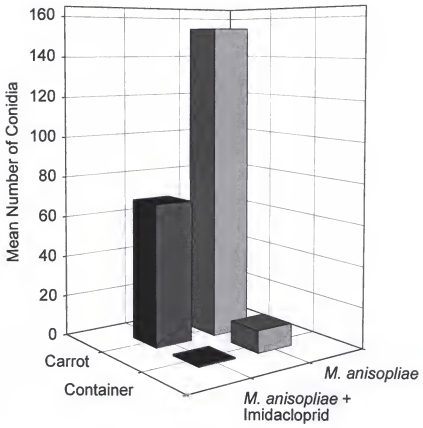

Fig. 3.6. Mean number of conidia of *Metarhizium anisopliae* attached to the carrots and plastic containers at 24 h post-treatment. Mean numbers of conidia on carrots and containers were estimated using dodine selective media in 40 and 37 replications for each treatment, respectively.

Conidial Attachment of *Metarhizium anisopliae* on Larval Cuticle Treated with Different Doses of Formulated Product and Each component Separately .

In test 1, conidial attachment to the larval cuticle decreased with an increase in doses of imidacloprid and best fit a second degree curve (R^2=0.61; df=2,37; P<0.0371) (Fig. 3.7). Only 88.0 conidia per microscope field were counted on the cuticle of larvae treated with imidacloprid at 1,000 ppm compared to 604.2 conidia for untreated larvae. Conidial attachment decreased 7-fold when doses of imidacloprid increased from 100 to 1,000 ppm, respectively.

In test 2, the active ingredient had no effect on conidial attachment compared to control (P<0.0859) (Fig. 3.8). However, the inert carrier and the formulated product affected conidial attachment negatively compared to control (P<0.0001 for both treatments). Only 71.2 and 126.9 conidia were found attached to larval cuticle for formulated product and inert carrier, respectively. These results showed that the inert carrier is the component responsible for lower conidial attachment on larval cuticle.

Conidial Attachment of *Metarhizium anisopliae* and *Beauveria bassiana* on Insect Cuticle in Soil Treated with Imidacloprid at Different Moistures.

M. anisopliae conidia attached to the larval cuticle regardless of the presence of imidacloprid (Fig. 3.9-A, C). The mean number of conidia attached to larvae cuticle following soil exposure to *M. anisopliae* at $5x10^5$ conidia/ml did not change among soil moistures (Fig. 3.9-A, Table 3.3). Conidial attachment following soil exposure to *M. anisopliae* at $5x10^6$ conidia/g was significantly higher at 0% compared to 7 and 14% soil moistures (3.9-B, Table 3.4). With the exception of soil at 0% moisture content, conidial attachment was higher in soil treated with the imidacloprid/fungus combination at $5x10^5$ and

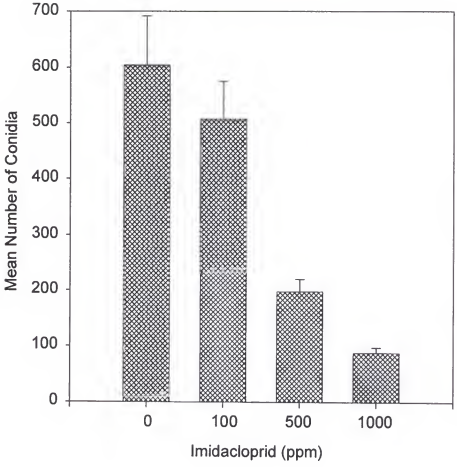

Fig. 3.7. Mean number of conidia of *Metarhizium anisopliae* on cuticle after cuticular exposure of first instar larvae of *Diaprepes abbreviatus* to different doses of imidacloprid.

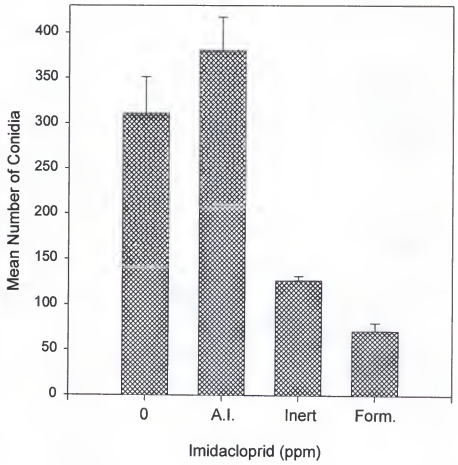

Fig. 3.8. Mean number of conidia of *Metarhizium anisopliae* on cuticle after cuticular exposure of first instar larvae of *Diaprepes abbreviatus* to active ingredient (A.I), inert carrier (Inert), and formulated product (Form.) .

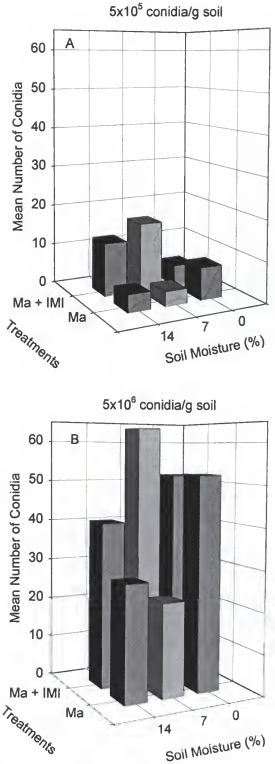

Fig. 3.9. Mean number of conidia of *Metarhizium anisopliae* on cuticle of first instar larvae of *Diaprepes abbreviatus* after exposure to soil treated with fungus at $5x10^5$ and $5x10^6$ conidia/g soil alone or in combination with imidacloprid at 25μg/g soil at different soil moistures.

Table 3.3. P values of the least square means analysis of the effect of conidial concentrations of *Metarhizium anisopliae* (Ma) applied alone at $5x10^5$ conidia/g soil to Candler soil or in combination with imidacloprid (IMI) on conidial germination on cuticle of first instar larvae of *Diaprepes abbreviatus* under 0, 7, and 14% soil moisture.

Treatment	Ma 0%	Ma 7%	Ma 14%	Ma+IMI 0%	Ma+IMI 7%	Ma+IMI 14%
Ma 0%	-	0.1828	0.2589	0.3767	0.0078	0.1200
Ma 7%	0.1828	-	0.8368	0.6505	0.0001	0.0046
Ma 14%	0.2589	0.8368	-	0.8047	0.0002	0.0082
Ma+IMI 0%	0.3767	0.6505	0.8047	-	0.0005	0.0160
Ma+IMI 7%	0.0078	0.0001	0.0002	0.0005	-	0.2504
Ma+IMI 14%	0.1200	0.0046	0.0082	0.0160	0.2504	-

Table 3.4. P values of the least square means analysis of the effect of conidial concentrations of *Metarhizium anisopliae* (Ma) applied alone at $5x10^6$ conidia/g soil to Candler soil or in combination with imidacloprid (IMI) on conidial germination on cuticle of first instar larvae of *Diaprepes abbreviatus* under 0, 7, and 14% soil moisture.

Treatment	Ma 0%	Ma 7%	Ma 14%	Ma+IMI 0%	Ma+IMI 7%	Ma+IMI 14%
Ma 0%	-	0.0191	0.0517	0.9775	0.3631	0.3382
Ma 7%	0.0191	-	0.6719	0.0211	0.0016	0.1530
Ma 14%	0.0517	0.6719	-	0.0565	0.0053	0.3105
Ma+IMI 0%	0.9775	0.0211	0.0565	-	0.3422	0.3589
Ma+IMI 7%	0.3631	0.0016	0.0053	0.3422	-	0.0650
Ma+IMI 14%	0.3382	0.1530	0.3105	0.3589	0.0650	-

$5x10^6$ conidia/g soil.

Conidial attachment ($P<0.040$) and larval mycosis ($P<0.0185$) for larval exposed to soil at 7% moisture with *B. bassiana* at $5x10^6$ conidia/g soil was significantly higher in imidacloprid-treated soil (Fig. 3.10).

Removal of *M. anisopliae* Conidia from the Larval Cuticle of *D. abbreviatus* Treated with Imidacloprid in Soil at Different Water Content.

Following application of *M. anisopliae* conidia to the cuticle, larvae removed 68.4-90.8% of the conidia during movement through untreated Candler soil at all soil moistures (Table 3.5). The number of conidia remaining on larval cuticle among insects exposed to untreated soil was highest at 0% compared to 7, and 14% water content. Higher conidial density on larvae exposed to 0% soil moisture suggests that they did not move or moved less in dry soil. When larvae were treated with imidacloprid suspension at 500 ppm, larval mobility was significantly impaired resulting in less conidial removal (0-2.8%). Larvae treated with imidacloprid before soil exposure had fewer conidia attached to the larval cuticle than the control suggesting reduced attachment caused by components of the chemical formulation.

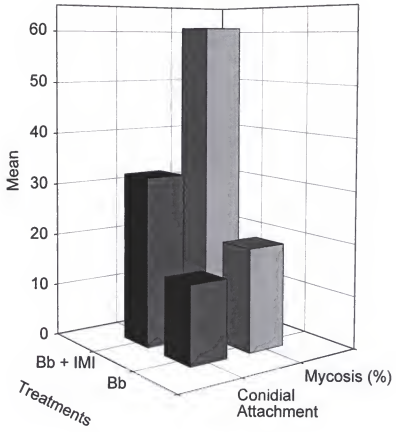

Fig. 3.10. Mean number of conidia of *Beauveria bassiana* on cuticle and larval mycosis of first instar larvae of *Diaprepes abbreviatus* after exposure to soil treated with fungus at $5x10^6$ conidia/g soil alone or in combination with imidacloprid at 25μg/g soil at 7% soil moistures.

Table 3.5. Effect of imidacloprid and soil moisture on the removal of *Metarhizim anisopliae* conidia from the larval cuticle of *Diaprepes abbreviatus*.

M. anisopliae (Conidia/ml)	Imidacloprid (ppm)	Soil moisture (%)	Mean no. of conidia (±SEM) on larval cuticle before soil exposure	Mean no. of conidia[a] (±SEM) on larval cuticle after soil exposure	Conidial removal (%)
10^7	0	0	380.0 ± 39.9	120.0 ± 14.7	68.4
		7	380.0 ± 39.9	49.9 ± 15.5	86.9
		14	380.0 ± 39.9	34.8 ± 13.0	90.8
10^7	500	0	46.6 ± 6.0	47.5 ± 6.2	0.0
		7	46.6 ± 6.0	45.3 ± 6.6	2.8
		14	46.6 ± 6.0	51.2 ± 8.1	0.0

[a] Means based on 15 microscopic fields per treatment examined at 630x.

CHAPTER 4
CONIDIAL GERMINATION ENHACEMENT OF *Metarhizium anisopliae* AND *Beauveria bassiana* WITH IMIDACLOPRID.

Introduction

Endoparasitic infections caused by *Beauveria bassiana* (Bals.) Vuill.and *Metarhizium anisopliae* (Metsch.) Sorokin in insects are initiated by the dry hydrophobic conidia (McCoy et al. 1988). Usually the conidia of these fungi will not swell and form germ tubes in distilled water. An exogenous nutrient is normally required to initiate germination (Dillon and Charnley 1985). Normally both *B. bassiana* and *M. anisopliae* require a carbon source for stimulation and growth of germ tubes (Smith and Grula 1981, Dillon and Charnley 1990).

The rate of conidial germination is usually associated with pathogenicity and differs from strain to strain. For example, rapid conidial germination of strains of *M. anisopliae* was correlated with higher pathogenicity to the brown plant hopper, *Nilaparvata lugens* (Samuels et al. 1989) and to mosquito larvae (Al-Aidroos and Roberts 1978, Daoust and Roberts 1982). Dillon and Charnley (1985) showed that soaking conidia of *M. anisopliae* in water for 24 h accelerates and synchronizes germination. Soaked conidia were also more pathogenic when tested against the caterpillar, *Manduca sexta* (Hassan et al. 1989).

Numerous *in vitro* and *in vivo* studies have been conducted to determine the effect of pesticides on the growth of *M. anisopliae* and *B. bassiana* (Osborne and Boucias 1988). In some of these studies, pesticides appeared to enhance germination (Keller 1978) and

promote mycelial growth (Sutton et al. 1979).

Preliminary studies suggested possible enhancement of conidial germination of *M. anisopliae* by imidacloprid. Accordingly, studies were initiated to determine the effect of imidacloprid on conidial germination of *M. anisopliae* and *B. bassiana* on both artificial substrates and on the insect cuticle of first instar larvae of *D. abbreviatus.*

Materials and Methods

General Procedures.

First instar larvae of *D. abbreviatus* and chemical used in these studies were the same as described in chapter 2. The fungal strains used and the production methodology were similar to those described in chapter 3.

Conidial Germination of *M. anisopliae* and *B. bassiana* on Water/Agar Media With and Without Imidacloprid.

Conidia of *M. anisopliae* were suspended in sterile 0.05% Tween 80 (Fisher Cientific). The conidial suspension was mixed in a vortex at high speed and then filtered through a cheese cloth to remove clumps and mycelial fragments. Conidial concentrations were determined using a bright line hemacytometer with a phase contrast microscope at 400x. Twenty ml of *M. anisopliae* at 10^7 conidia/ml were added with imidacloprid at 0, 1, 10, 100, 1,000, or 10,000 ppm a.i. A 200-μl aliquot of each suspension was pipetted onto 90mm-Petri dish containing water/agar and then spread over the surface of the medium with a bent glass rod. The plates were incubated at 26^{0}C. At 10, 12, 14, and 16 h post-inoculations, conidial viability were determined using a Zeiss phase microscope. Two plates for each treatment were counted per time interval. A minimum of 250 conidia were counted

in five replications. Using the same procedure, a second test was conducted to determine the effect of imidacloprid at 0, 100, 1,000, or 10,000 ppm ai on conidial germination of *B. bassiana* at 8, 10, and 12 h post-inoculation. Bioassay data were transformed to arcsine $\sqrt{x}$ before performing regression analysis using the general linear model procedure to determine the relationship between conidial germination and imidacloprid concentration (SAS Institute 1985).

Conidial Germination of *M. anisopliae* and *B. bassiana* on SDAy Media With and Without Imidacloprid.

The effect of imidacloprid at 0, 100, 1,000, and 10,000 ppm ai on conidial germination of *M. anisopliae* and *B. bassiana* was also evaluated in Sabouraud dextrose agar plus 0.4% yeast extract (SDAy). Conidial germination was determined at 8, 10, and 12 h post-inoculation. Other experimental procedures and regression analysis followed methodology described above.

Conidial Germination of *M. anisopliae* on Water Agar Media With Formulated Imidacloprid and Each Component Separately.

Formulated imidacloprid and active ingredient at 100, 1,000 and 10,000 ppm and inert carrier at 367, 3,670, and 36,700 ppm (concentration equal to the amount found in formulated imidacloprid at 100, 1,000, and 10,000 ppm of the active ingredient, respectively) were mixed with conidia of *M. anisopliae* at concentrations of 0, and 10^7 conidia/ml. A 200-μl aliquot of each suspension was pipetted onto 90mm-Petri dish containing water/agar media to determine the effect of these components on conidial germination at 10, 13, and 16 h post-inoculation.

Conidial Germination of *M. anisopliae* on Water Agar Media With Each Component of the Inert Carrier.

The effect of each component of the inert carrier was tested to determine their effect on conidial germination of *M. anisopliae* on water/agar media at 18 h post-inoculation. The actual name of each component is not identified for proprietary reasons. A minimum of 400 total conidia were examined in 4 replications.

Conidial Germination of *M. anisopliae* and *B. bassiana* on Imidacloprid Treated Larval Cuticle of *D. abbreviatus.*

Conidia of *M. anisopliae* and *B. bassiana* were suspended in 0.05 M carbonate - bicarbonate buffer, pH 9.2 in Tween 80 0.01%. The suspensions were vortexed at high speed and then filtered through a cloth screen and sequentially washed in distilled sterilized water by centrifugation at 1,000 relative centrifugal force (RCF) for 5 min. The conidia were stained with 1 mg of fluorescein isothiocyanate (FITC, Sigma Chem.) in 1 ml of carbonate-bicarbonate buffer for 1 hr at room temperature according to Hung and Boucias (1992). Stained conidia were washed five times with sterile distilled water prior to being used in the experiments.

A 1.5 ml suspension containing FITC-labeled conidia of *M. anisopliae* or *B. bassiana* at 10^8 conidia/ml were mixed alone or in combination with imidacloprid at a concentration of 500 ppm to 30 g of Candler soil. These concentrations were equivalent to fungus at $5x10^6$ conidia/g soil and chemical at 25 μg a.i./g soil, respectively. Soil moisture was adjusted to 7 and 14% by adding sterile distilled water and to 0% moisture by air-drying treated soil in a dark incubator at 28^{0}C with continuous air flow. For the *B. bassiana* experiment, soil moisture was held constant at 7% water content. Two g of treated soil were added to a depth

of 1 cm to bioassay columns (15 cm ht, 2.0 cm diam.) constructed from polystyrene tube and attached to a containment cell (Hamlen and Beavers 1975). Twenty 48-h-old first instar larvae were placed on the soil surface in each container. Bioassay units were closed tightly and held at 28° C. Each treatment was replicated 3 times. After 22 h, larvae recovered from each containment cell and the soil were mounted in 1,4-diazabicyclo[2.2.2]octane. Conidial germination on larval cuticle was examined at 1,000 magnification using an epifluorescent Leica microscope using an exciter filter BP 450-490 nm and a barrier filter of 0-530 nm. Photographs were made using a TMAx 400 ASA 35-mm film. For each treatment, between 250-350 conidia were randomly selected for examination on the cuticle of 10 larvae. Larval mycosis was also recorded after 5 days. Twenty first instar larvae were tested for each treatment in 3 replications. The number of dead and alive insects recovered from the containment cell and the soil in each column was determined microscopically at 16x. Dead larvae were held in 35 mm Petri dishes containing a 1.5 mm moistened filter paper to determine mycosis by *M. anisopliae* or *B. bassiana*.

Examination of the Larval Cuticle Treated With Imidacloprid for Conidial Germination of *M. anisopliae* Using Scanning Electron Microscope.

To determine the effect of imidacloprid on conidial germination on larval cuticle of *D. abbreviatus*, the chemical was administered by contact or oral exposure in combination with *M. anisopliae* conidia. Approximately 50 first instar larvae were placed in 1.5 ml micro-centrifuge tubes containing imidacloprid at 200 ppm and *M. anisopliae* at 10^7 conidia/ml prepared in 0.05% aqueous Tween 80. The tubes were agitated gently for 30 seconds before larval removal. Fungus-treated larvae were treated orally with imidacloprid

by feeding them continuously on carrot, previously soaked in the imidacloprid at 200 ppm for 30 min. Larvae were held in 30-ml plastic cups. Larvae examined by scanning electron microscopy were fixed at 0, 12, and 24 h after treatment using two methods. In the first method, Hallam's variation of Karnovsky's was used (Hallam 1970). In the second method, two fixative solutions were used as described by Grodowitz et al. (1982). The larvae were then coated with gold/palladium (80:20) and examined with a Hitachi S-530 scanning electron microscope operating at 20 kV. Photographs were taken with polaroid 55 film.

Statistical analysis.

All bioassays data were analyzed using Proc GLM and treatment means compared using proc LSMEANS(Least square means) unless stated otherwise (SAS Institute 1985).

Results

Conidial Germination of *M. anisopliae* and *B. bassiana* on Water/Agar Media With and Without Imidacloprid.

The rate and number of conidia of *M. anisopliae* germinating on water agar after 16 h increased 4-fold with an increase in chemical concentration from 1 to 10,000 ppm (Fig. 4.1). At 10 and 12 h post-inoculation, conidial germination was higher at concentrations above 100 ppm compared to the control and the relatonship between germination and chemical rate best fit a 3rd degree equation for 10 (R^2=0.96; F=20.92; df=3,26; P<0.0001) and 12 h (R^2=0.96; F=126.40; df=3,26; P<0.0001). At 14 h post-treatment, conidial germination of *M. anisopliae* were detected at concentrations as low as 1 ppm and the relationship was best described by a second degree curve (R^2=0.93, F=154.84; df=2,26; P<0.0001). All concentrations of imidacloprid affected the rate and number of conidia of *M.*

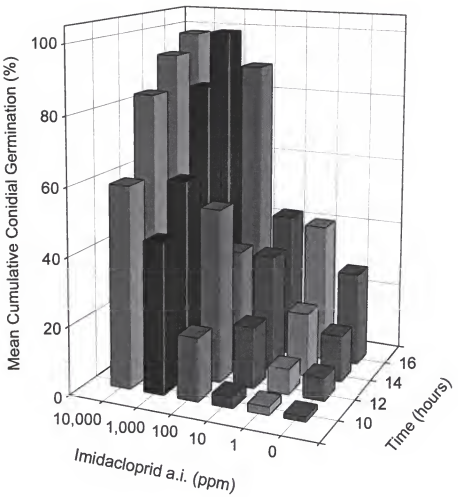

Fig. 4.1. Effect of different concentrations of imidacloprid on rate of conidial germination of *Metarhizium anisopliae* on surface of water agar media in time.

anisopliae germinating at 16 h post-inoculation. The relationship between imidacloprid concentration and conidial germination was highly significant and best fit a third degree curve (R^2=0.97; F=280.93; df=3,26; P<0.0001).

Imidacloprid had no effect on conidial germination of *B. bassiana* on water agar at 8 (r^2=0.15, F=0.13; df=1,13; P>0.7249), 10 (r^2=0.32, F=0.45; df=1,13; P>0.5144), and 12 h (r^2=0.09, F=0.03; df=1,13; P>0.8611), probably because conidial germination occurred on this low nutritious medium (Fig. 4.2). Conidia of *B. bassiana* germinated much faster and in greater numbers compared to *M. anisopliae*. For example at 10 h, 89% of the *B. bassiana* conidia germinated compared to 1.4% for *M. anisopliae* (Figs. 4.1, 4.2).

Conidial Germination of *M. anisopliae* and *B. bassiana* on SDAy Media With and Without Imidacloprid.

On SDAy, a highly nutritious medium, conidial germination of *M. anisopliae* appears to be inhibited as concentration of imidacloprid increased from 100 to10,000 ppm (Fig. 4.3-A) and decreased linearly as doses of imidacloprid increased at 8 (r^2=0.67; F=23.67; df=1,13; P<0.0004) 10 (r^2=0.54; F=11.67; df=1,13; P<0.0051) and at 12 h post-treatment (r^2=0.21; F=4.41; df=1,13; P<0.0574).

For *B. bassiana*, conidial germination at 8 h increased as the concentrations of imidacloprid increased and best fit a third degree equation (R^2=0.76; F=4.71; df=3,12; P<0.0509) (Fig. 4.3 B). However, imidacloprid had no effect on conidial germination of *B. bassiana* at 10 (r^2=0.21; F=4.02; df=1,13; P<0.0681) and 12 h (r^2=0.003; F=0.04; df=1,13; P>0.8529). Conidial germination of *B. bassiana* on SDAy was faster compared to *M. anisopliae*.

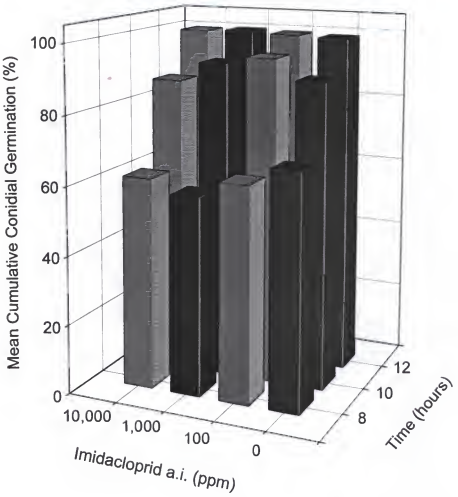

Fig. 4.2. Effect of different concentrations of imidacloprid on rate of conidial germination of *Beauveria bassiana* on surface of water agar media in time.

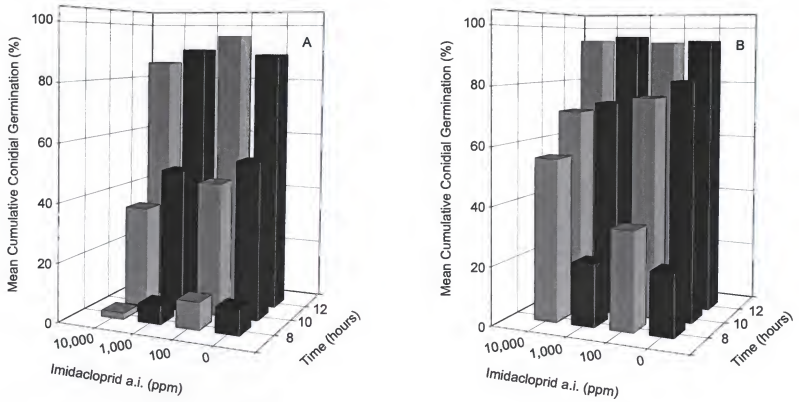

Fig. 4.3. Effect of different concentrations of imidacloprid on rate of conidial germination of *Metarhizium anisopliae* (A) and *Beauveria bassiana* (B) on surface of SDAy media in time.

Conidial Germination of *M. anisopliae* on Water Agar Media With Formulated Imidacloprid and Each Component Separately.

The active ingredient at 100 ppm had no effect on conidial germination of *M. anisopliae* compared to control in time (Fig 4.4-A, Tables 4.1, 4.2, 4.3). However, conidial germination was significantly enhanced by the active ingredient at 1,000 ppm after 10 h and at 10,000 ppm (Fig 4.4-B, C). The formulated product and the inert carrier had a significantly greater effect on conidial germination compared to the active ingredient at all concentrations (Fig. 4.4, Tables 4.1, 4.2, 4.3). The rate of conidial germination of *M. anisopliae* was significantly increased by the inert carrier compared to the formulated product at 100 and 1,000 ppm. However, germination was similar for the inert carrier and formulated product at all concentrations at 16 h.

Conidial Germination of *M. anisopliae* on Water Agar Media With Each Component of the Inert Carrier.

Component A was the most important ingredient of the inert carrier for stimulating germination of *M. anisopliae* conidia (Fig. 4.5, Table 4.4). Componet B also significantly increased germination compared to the control. When the concentration of componen A was increased from 1,000 to 10,000 ppm, conidial germination of *M. anisopliae* was not affected ($P>0.9164$). No conidial germination was observed for component C at 1,000 and 10,000 ppm. This compound has also significantly impaired mycelial growth of *M. anisopliae* at higher concentrations (Quintela and McCoy unpublished).

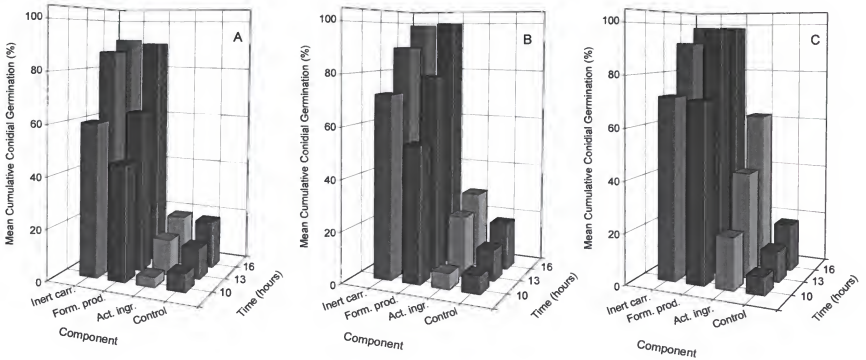

Fig. 4.4. Effect of formulated imidacloprid and active ingredient at 100 ppm and inert carrier at 367 ppm (A) (concentration equal to the amount found in formulated imidacloprid at 100 ppm ai), formulated imidacloprid and active ingredient at 1,000 ppm and inert carrier at 3,670 ppm (B), formulated imidacloprid and active ingredient at 10,000 ppm and inert carrier at 36,700 ppm (C) on conidial germination of *Metarhizium anisopliae* on surface of water agar media in time.

Table 4.1. P values of the least square means analysis (LSMEANS) of the effect of formulated imidacloprid (FP) and active ingredient (AI) at 100, 1,000, and 10,000 ppm and inert carrier (IC) at 367 ppm, 3,670, and 36,700 ppm (concentration equal to the amount found in formulated imidacloprid at 100 ppm a.i.) on conidial germination of *Metarhizium anisopliae* at 10 h.

Treatments	0	AI 100	AI 1,000	AI 10,000	IC 367	IC 3,670	IC 36,700	FP 100	FP 1,000	FP 10,000
0	-	0.5767	0.8811	0.0204	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 100	0.5767	-	0.6821	0.0052	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 1,000	0.8811	0.6821	-	0.0144	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 10,000	0.0204	0.0052	0.0144	-	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
IC 367	0.0001	0.0001	0.0001	0.0001	-	0.0377	0.0429	0.0089	0.2113	0.0667
IC 3,670	0.0001	0.0001	0.0001	0.0001	0.0377	-	0.9530	0.0001	0.0017	0.7882
IC 36,700	0.0001	0.0001	0.0001	0.0001	0.0429	0.9530	-	0.0001	0.0020	0.8338
FP 100	0.0001	0.0001	0.0001	0.0001	0.0089	0.0001	0.0001	-	0.1390	0.0001
FP 1,000	0.0001	0.0001	0.0001	0.0001	0.2113	0.0017	0.0020	0.1390	-	0.0034
FP 10,000	0.0001	0.0001	0.0001	0.0001	0.0667	0.7882	0.8338	0.0001	0.0034	-

Table 4.2. P values of the least square means (LSMEANS) analysis of the effect of formulated imidacloprid (FP) and active ingredient (AI) at 100, 1,000, and 10,000 ppm and inert carrier (IC) at 367 ppm, 3,670, and 36,700 ppm (concentration equal to the amount found in formulated imidacloprid at 100 ppm a.i.) on conidial germination of *Metarhizium anisopliae* at 13 h.

Treatments	0	AI 100	AI 1,000	AI 10,000	IC 367	IC 3,670	IC 36,700	FP 100	FP 1,000	FP 10,000
0	-	0.7232	0.0193	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 100	0.7232	-	0.0428	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 1,000	0.0193	0.0428	-	0.0008	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 10,000	0.0001	0.0001	0.0008	-	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
IC 367	0.0001	0.0001	0.0001	0.0001	-	0.5674	0.3095	0.0001	0.0797	0.0337
IC 3,670	0.0001	0.0001	0.0001	0.0001	0.5674	-	0.6520	0.0001	0.0232	0.1099
IC 36,700	0.0001	0.0001	0.0001	0.0001	0.3095	0.6520	-	0.0001	0.0079	0.2426
FP 100	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	-	0.0025	0.0001
FP 1,000	0.0001	0.0001	0.0001	0.0001	0.0797	0.0232	0.0079	0.0025	-	0.0003
FP 10,000	0.0001	0.0001	0.0001	0.0001	0.0337	0.1099	0.2426	0.0001	0.0003	-

Table 4.3. P values of the least square means (LSMEANS) analysis of the effect of formulated imidacloprid (FP) and active ingredient (AI) at 100, 1,000, and 10,000 ppm and inert carrier (IC) at 367 ppm, 3,670, and 36,700 ppm (concentration equal to the amount found in formulated imidacloprid at 100 ppm a.i.) on conidial germination of *Metarhizium anisopliae* at 16 h.

Treatments	0	AI 100	AI 1,000	AI 10,000	IC 367	IC 3,670	IC 36,700	FP 100	FP 1,000	FP 10,000
0	-	0.8334	0.0025	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 100	0.8334	-	0.0043	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 1,000	0.0025	0.0043	-	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
AI 10,000	0.0001	0.0001	0.0001	-	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
IC 367	0.0001	0.0001	0.0001	0.0001	-	0.0421	0.0581	0.6935	0.0227	0.0585
IC 3,670	0.0001	0.0001	0.0001	0.0001	0.0421	-	0.8794	0.0173	0.7826	0.8769
IC 36,700	0.0001	0.0001	0.0001	0.0001	0.0581	0.8794	-	0.0245	0.6693	0.9974
FP 100	0.0001	0.0001	0.0001	0.0001	0.6935	0.0173	0.0245	-	0.0089	0.0247
FP 1,000	0.0001	0.0001	0.0001	0.0001	0.0227	0.7826	0.6693	0.0089	-	0.6670
FP 10,000	0.0001	0.0001	0.0001	0.0001	0.0585	0.8769	0.9974	0.0247	0.6670	-

Table 4.4. P values of the least square means (LSMEANS) analysis of the effect of different concentrations of the components of the inert carrier as proxel (P), morwet (M), block copolumer (BC), rhodopol (R), and glycerine (G) on conidial germination of *Metarhizium anisopliae* on surface of water agar media.

Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
BC 100	0.6241	G 100	0.0001	M 100	0.7456	P 100	0.7288	R 100	0.0004
BC 1,000	0.8857	G 1,000	0.0001	M 1,000	0.1282	P 1,000	0.1977	R 1,000	0.0289
BC 10,000	0.6597	G 10,000	0.0001	M 10,000	0.2233	P 10,000	0.1739	R 10,000	0.0001

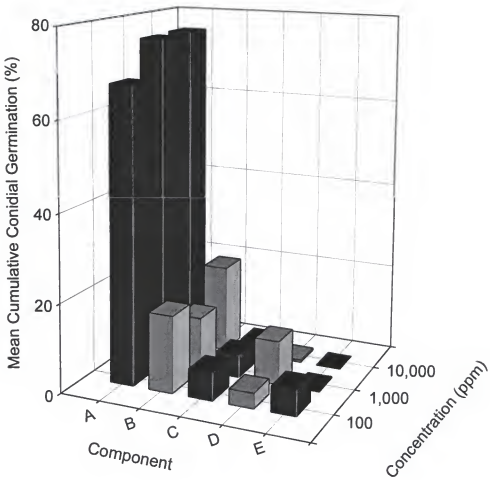

Fig. 4.5. Effect of different concentrations of the components of the inert carrier on conidial germination of *Metarhizium anisopliae* on surface of water agar media at 18 h post-inoculation.

Conidial Germination of *M. anisopliae* and *B. bassiana* on Imidacloprid Treated Larval Cuticle of *D. abbreviatus.*

Conidial germination of *M. anisopliae* on larval cuticle of *D. abbreviatus* at 22 h increased as soil moisture increased from 0-14% (Fig. 4.6-A, Table 4.5). At 0% soil moisture, germination was similar for *M. anisopliae* applied alone or in combination with imidacloprid. At 7 and 14% moisture conidial germination was significantly higher for the fungus/imidacloprid interaction compared to the fungus alone (Fig. 4.6-A, Table 4.5). Although conidial germination on insects held at 0% moisture was very low, larval mycosis after 5 days was significantly higher for *M. anisopliae* alone compared to 7 and 14% moistures (Fig. 4.6-B, Table 4.6). When imidacloprid was included with *M. anisopliae* conidial suspension and applied to the soil, larval mycosis increased significantly. For example, larval mycosis for fungus alone was 56.0, 33.3, and 29.6% compared to 97.2, 92.4, and 62.7% for the fungus/chemical combination at 0, 7, and 14% soil moisture, respectively. Larval mycosis for the fungus/chemical combination was significantly lower at 14% compared to 0 and 7%.

Conidial germination ($P<0.0001$) and larval mycosis ($P<0.0185$) on larval cuticle in soil at 7% moisture treated with *B. bassiana* at $5x10^6$ conidia/g soil were significantly higher in imidacloprid-treated soil (Fig. 5.7).

Examination of the Larval Cuticle Treated With Imidacloprid for Conidial Germination of *M. anisopliae* Using Fluorescence and Scanning Electron Microscopy.

When Hallam's variation of Karnovsky fixative (Hallam 1970) was used to fix first instar larvae of *D. abbreviatus*, specimens collapsed and many morphological features were distorted due to poor fixative penetration (Fig. 4.8 A). However, using the fixation method

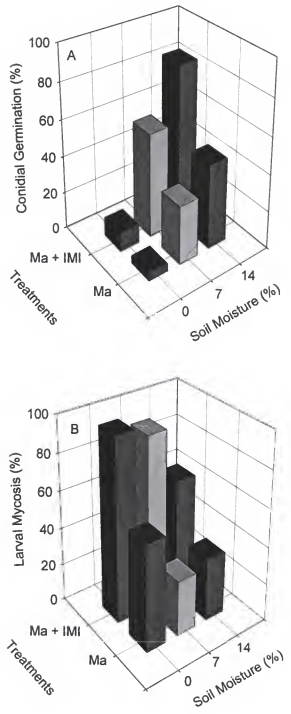

Fig. 4.6. Effect of conidial concentrations of *Metarhizium anisopliae* applied alone to Candler soil or in combination with imidacloprid on conidial germination on larval cuticle at 22 h (A), and on larval mycosis after 5 d (B) of first instar larvae of *Diaprepes abbreviatus* under different soil moistures.

Table 4.5. P values of the least square means (LSMEANS) analysis of the effect of conidial concentrations of *Metarhizium anisopliae* (Ma) applied alone to Candler soil or in combination with imidacloprid (IMI) on conidial germination on cuticle of first instar larvae of *Diaprepes abbreviatus* under 0, 7, and 14% soil moisture

.

Treatment	Ma 0%	Ma 7%	Ma 14%	Ma+IMI 0%	Ma+IMI 7%	Ma+IMI 14%
Ma 0%	-	0.0561	0.0105	0.6935	0.0011	0.0001
Ma 7%	0.0561	-	0.3030	0.1320	0.0595	0.0003
Ma 14%	0.0105	0.3030	-	0.0264	0.4511	0.0105
Ma+IMI 0%	0.6935	0.1320	0.0264	-	0.0033	0.0001
Ma+IMI 7%	0.0011	0.0595	0.4511	0.0033	-	0.0448
Ma+IMI 14%	0.0001	0.0003	0.0105	0.0001	0.0448	-

Table 4.6. P values of the least square means analysis of the effect of conidial concentrations of *Metarhizium anisopliae* (Ma) applied alone to Candler soil or in combination with imidacloprid (IMI) on mycosis of first instar larvae of *Diaprepes abbreviatus* under 0, 7, and 14% soil moisture.

Treatment	Ma 0%	Ma 7%	Ma 14%	Ma+IMI 0%	Ma+IMI 7%	Ma+IMI 14%
Ma 0%	-	0.0372	0.0441	0.0038	0.0041	0.5087
Ma 7%	0.0372	-	0.7391	0.0002	0.0002	0.0130
Ma 14%	0.0441	0.7391	-	0.0002	0.0001	0.0136
Ma+IMI 0%	0.0038	0.0002	0.0002	-	0.6715	0.0106
Ma+IMI 7%	0.0041	0.0002	0.0001	0.6715	-	0.0129
Ma+IMI 14%	0.5087	0.0130	0.0136	0.0106	0.0129	-

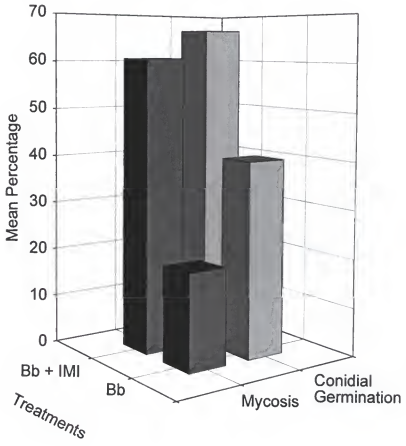

Fig. 4.7. Effect of conidial concentrations of *Beauveria bassiana* applied alone to Candler soil or in combination with imidacloprid at 25μg/soil on conidial germination on larval cuticle at 22 h post-treatment, and on larval mycosis after 5 d of first instar larvae of *Diaprepes abbreviatus* at 7% soil moisture.

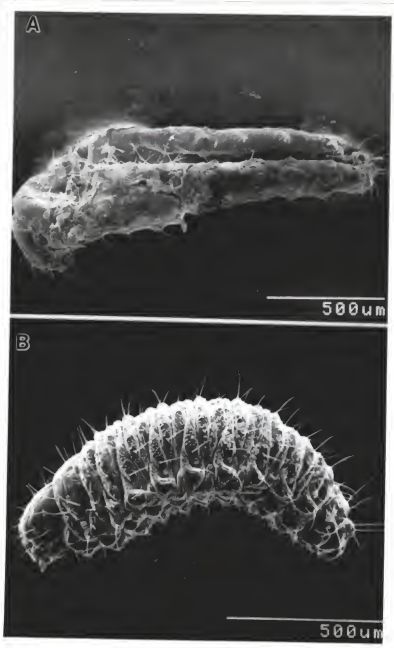

Fig. 4.8. Lateral view of first instar larvae of *Diaprepes abbreviatus* A) Larva fixed with a Hallam's variation of Karnovsky's fixative. B) Larva fixed according methodology of Grodowitz et al (1982).

of Grodowitz et al (1982) for soft bodied larval Diptera, excellent fixation was achieved with no noticeable collapse or shrinkage of the cuticle (Fig. 4.8 B). This method also gave excellent fixation of *M. anisopliae* conidia (Fig. 4.9).

Conidial germination and germ tube penetration through the cephalic capsule by *M. anisopliae* occurred within 12 h after cuticular treatment with imidacloprid at 200 ppm (Fig 4.9 A). The germ tube differentiated to an appressorium and secreted copious mucilage at the site of penetrating the cephalic capsule of the larvae. No germination occurred on larval cuticle treated with only wetting agent. After 24 h, conidial germination by *M. anisopliae* was observed on the cuticle of larvae treated with imidacloprid (contact or oral exposure) and untreated cuticle (Figs 4.9-B, C, D). The germ tubes on larval cuticle treated with conidia of *M. anisopliae* only orientated to the membranous region surrounding cuticular setae (Fig. 4.9 C). When treated with chemical, the germ-tube formed a spiral to facilitate penetration into larval integument (Fig. 4.9 F). Mucilage secretion was observed terminally on the germ-tube (Fig. 4.9 F).

Germ-tubes of *M. anisopliae* were detected easily using FITC labelling technique (Fig. 4.10). However, the site of conidial germination of *B. bassiana* appears faint where the germ-tube formed (Fig. 4.10 B).

Normally the germ tube of *M. anisopliae* grew in one direction until altered by some irregularity on the larval cuticle such as sensilla (Fig. 4.10 A, D, E). Many times, the germ tube vanished into intersegmental folds (Figs. 4.9 E, 4.10 H). Germ tubes arising from individual conidia frequently branched to form hyphae (Fig. 4.9 E).

Generally, one germ-tube arose from each conidium, usually slightly oblique to one

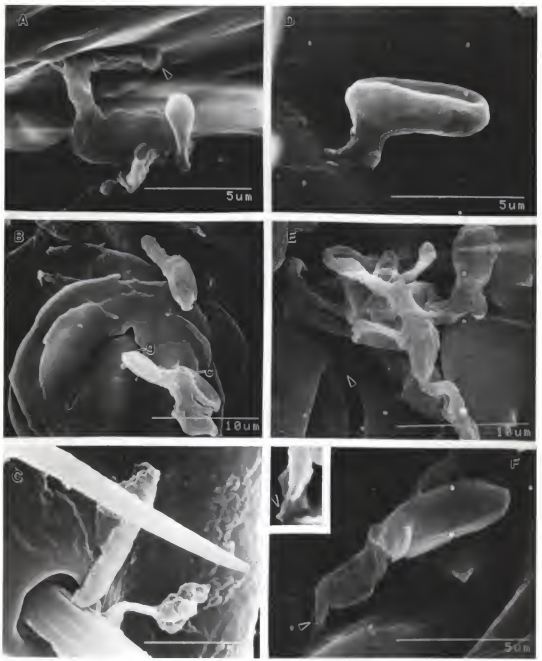

Fig. 4.9. Scanning electron micrographs of conidia of *Metarhizium anisopliae* on cuticle of first instar larvae of *Diaprepes abbreviatus*. A) Conidium germination and penetration on cephalic capsule of larva that were treated by cuticular exposure with imidacloprid at 200 ppm ai 12 h post-treatment. B) Conidia germination on the spiracle cuticular region of larvae immersed in imidacloprid at 200 ppm a.i at 12 h. C- conidium, g- germ-tube. C) Germ-tube orientation to membranous region surrounding cuticular setae at 24 h. D) Conidium germination and penetration on cuticle of larva treated orally with imidacloprid at 200 ppm ai and fixed at 24 h. E) Germ-tubes arising from individual conidia branched to form hyphae on larval cuticle treated by contact exposure with imidacloprid at 200 ppm ai at 24 h. F) Conidium on cuticle of larvae treated by contact exposure with imidacloprid at 200 ppm ai. Note production of mucilage terminally on germ-tube (arrows).

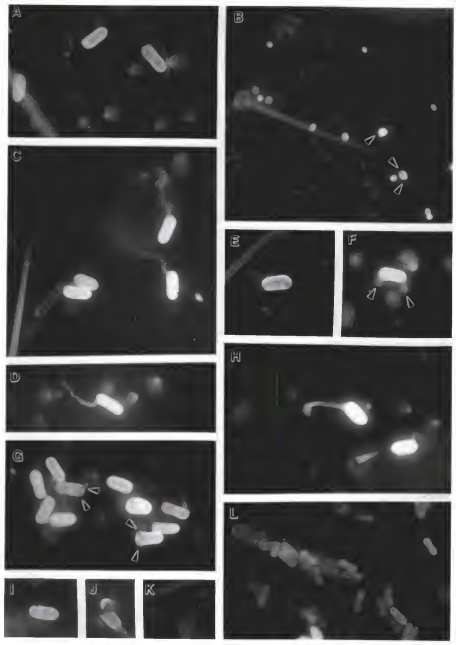

Fig. 4.10. Epifluorescence photomicrograph of FITC labeled conidia of *Metarhizium anisopliae* on larval cuticle of *Diaprepes abbreviatus* held in soil treated with imidacloprid at 25μg a.i./g soil at 22 h postapplication. A, D, E) Germ-tubes changed direction after approaching sensilla on larval cuticle. B) Germinated and ungerminated conidia of *B. bassiana*. Note the faint outline where germ-tubes were formed (arrows). C) Germ-tubes of different width. F) Bipolar germ-tube (arrow). G) Unipolar and bipolar germ-tubes. H) Terminally appressorium on germ tube on intersegmental fold of larval cuticle. I, J, K) Conidia with the cell wall disrupted. L) Conidia in different stages of development on insect cuticle.

of the poles (Fig. 4.10 A, C, D, E). Bipolar germination was not uncommon on imidacloprid treated larvae by contact exposure (Figs. 4.10 F, G). Germ-tubes of different width were observed on chemically treated larvae by contact (Fig. 4.10 C). In some cases, the conidia cell wall was disrupted, but no germ-tube formation was observed from these cells (Fig. 4.10 I, J, K).

Appressoria usually developed at the ends of short-germ tube (Fig. 4.9 A) or terminally on moderately long germ-tube (Fig. 4.10 H). However, the germ-tube penetrated the larval cuticle directly without differentiating to an appressorium (Fig. 4.9 D). The depression on the conidial surface was the result of a loss of cell contents from the conidium at the time of germ-tube formation. Variations in the germination process for *M. anisopliae* were observed at the same time in a group of conidia (Fig. 4.10 L).

CHAPTER 5
PATHOGENICITY ENHANCEMENT OF *Metarhizium anisopliae* AND *Beauveria bassiana* TO LARVAE OF Diaprepes abbreviatus(COLEOPTERA:CURCULIONIDAE) WITH SUBLETHAL DOSES OF IMIDACLOPRID.

Introduction

In view of the physiological effects of imidacloprid on first instar larvae of *D. abbreviatus* and on conidial germination and its implication as a behavior-modifier and/or potential synergist to the infection process of entomopathogenic fungi, laboratory studies were conducted to determine the effect of sublethal concentrations of imidacloprid and different concentrations of fungal conidia on larval mortality, mycosis, and ecdysis. This chapter addresses the direct interaction of the fungi, *B. bassiana* and *M. anisopliae* with imidacloprid on first instar larvae of *D. abbreviatus*.

Materials and Methods

General Procedures

The insects, chemical, and fungi were the same as reported in chapter 2 and3.

Cuticular and Oral Treatment of First Instar Larvae with Imidacloprid and Conidia of *M. anisopliae* and *B. bassiana*.

In test 1, approximately 50 first instar larvae were placed in 1.5 ml micro-centrifuge tubes containing imidacloprid alone at 0 and 100 ppm ai, or conidia of *M. anisopliae* alone at concentrations of 0, 10^4, 10^5, 10^6, and 10^7 conidia/ml, and in combination as conidial suspensions. Aqueous Tween 80 0.05% was included with conidial and chemical mixtures

and served as a control. The tubes were agitated gently for 30 seconds before larval removal. Each treatment was replicated six times with six larvae per replicate. Larvae were held at 28 °C in the dark in 30 ml-clear plastic cups containing a 1.5 cm dia. filter paper disc moistened with sterile distilled water. A thin slice of raw carrot was supplied as a food source. The filter paper was moistened every other day and the carrots replaced when they began to deteriorate. Larval mortality was recorded daily by microscopic examination at 10-16x, starting on day two post-treatment. Dead larvae were removed daily and placed into 35-mm Petri dishes containing moistened filter paper to determine mycosis from *M. anisopliae*. In test 2, *B. bassiana* was substituted for *M. anisopliae* as the fungal source and each treatment was replicated five times with 10 larvae per replication.

In a series of bioassays (tests 3, 4, and 5), imidacloprid was tested at: 1) concentrations of 0, 25, 50, 100, 150, and 200 ppm, with conidial suspensions of *B. bassiana* at 0, and 10^6 conidia/ml alone and in combination, 2) concentrations of 0, 100, 250, 500, and 1000 ppm with conidial concentrations of *M. anisopliae* at 0, and 10^7 conidia/ml alone, and the combination, 3) concentrations of 0, 100, 500, and 1000 ppm with conidial suspensions of *B. bassiana* at 0, 10^5, 10^6, and 10^7 conidia/ml alone and in combination. For each test, each treatment was replicated five times with 10 larvae per replication. Larval mortality was recorded daily by microscopic examination. In addition, larval ecdysis was recorded daily for test 5.

To determine the effect of oral and cuticular exposure on larval mortality, mycosis, and ecdysis (test 6), imidacloprid was administered two ways, at concentrations of 0 and 100 ppm, and as conidial suspensions of *M. anisopliae* at concentrations of 0, 10^5, 10^6, and 10^7

conidia/ml alone and in combination. Larvae were treated orally by feeding them continuously on carrot, previously soaked in the imidacloprid solutions for 30 min. Larvae were treated by contact through dipping. Larvae were held in plastic cups containing an 8-mm dia. carrot slice. Each treatment was replicated five times with 10 larvae per replication. Diagnosis for larval mortality and mycosis were the same as described above.

A factorial analysis was performed on bioassay data to determine the main effects of imidacloprid and fungal treatments alone and their interaction (nonadditivity). Factorial analysis were performed using arsine √proportion mortality or mycosis transformed data using general linear model procedure (Proc GLM) (SAS Institute, 1985). Time-mortality regression analysis for some tests was performed by probit analysis ignoring the correlation in the data (SAS Institute, 1985). Failure of 95% CI overlap was used as the criterion to determine significant differences among treatments means.

Cuticular Exposure to Formulated Imidacloprid, and to Each Component Separately with Conidia of *M. anisopliae*.

Formulated imidacloprid at 500 ppm, active ingredient (ai) at 500 ppm, and inert carrier at 1,836 ppm (concentration equal to the amount found in formulated imidacloprid at 500 ppm of the active ingredient) were mixed with conidia of *M. anisopliae* at concentrations of 0, and 10^7 conidia/ml. Larvae were dipped in each suspension in the manner described previously. Aqueous Tween 80 at 0.05% was used as control. Treatments were replicated five times, with 10 larvae per replication. Larval mortality was recorded daily. Mortality and mycosis data were analyzed using Proc GLM after transformion to arsine √proportion. Treatment means were compared using Tukey's Honestly Significant

Difference (HSD) test at P=0.05 (SAS Institute 1985).

Results

Cuticular and Oral Treatment of First Instar Larvae with Imidacloprid and Conidia of *M. anisopliae* and *B. bassiana.*

Comparison between *M. anisopliae* and *B. bassiana.* According to the factorial analysis for tests 1 and 2, *M. anisopliae*, *B. bassiana*, and imidacloprid had a significant effect on larval mortality (Table 5.1). As shown in Fig. 5.1-D, H, larval mortality caused by cuticular exposure to imidacloprid at 100 ppm ranged from 18-30% compared to 10-18% in the untreated controls in test 1 and 2, respectively. In test 2, slightly higher mortality was probably the result of accidental fungal contamination (Fig. 5.1-F). Larval mortality and larval mycosis increased slowly with an increase in conidial concentration of both *M. anisopliae* and *B. bassiana* (Fig. 5.1-B, D, F, H). In fact, no larval mycosis from *M. anisopliae* occurred at conidial concentrations of 10^4 and 10^5 conidia/ml (Fig. 5.1-B).

When a sublethal dose (100 ppm) of imidacloprid was included with different concentrations of both *M. anisopliae* (test 1) and *B. bassiana* (test 2) and applied directly to the larval cuticle, there was a significant interaction (nonadditivity) between imidacloprid and fungal concentrations for both larval mortality and larval mycosis (Table 5.1). Synergism between the fungi and chemical is indicated by the significance of the main effects, the significance of the interaction, and the increased differences in larval mortality or mycosis between fungus alone compared to the combination (Fig. 5.1 A, C, E, G).

Table 5.1. Summary of factorial analysis for larval mortality and mycosis of *Diaprepes abbreviatus* subsequent to treatment with *Beauveria bassiana* (Bb) or *Metarhizium anisopliae* (Ma) and imidacloprid alone or in combination.

Test	Factor	Larval mortality[a]			Larval mycosis[a]		
		F	df	P value	F	df	P value
1	Ma	22.96	4,50	0.0001	75.56	4,50	0.0001
	Chemical	45.93	1,50	0.0001	92.39	1,50	0.0001
	FxC	2.62	4,50	0.0456	10.21	4,50	0.0001
2	Bb	14.59	4,40	0.0001	25.20	4,40	0.0001
	Chemical	63.42	1,40	0.0001	63.45	1,40	0.0001
	FxC	4.17	4,40	0.0065	4.58	4,40	0.0039
3	Bb	43.84	1,48	0.0001	412.84	1,48	0.0001
	Chemical	15.62	5,48	0.0001	18.69	5,48	0.0001
	FxC	7.15	5,48	0.0001	18.41	5,48	0.0001
4	Ma	90.46	1,40	0.0001	336.82	1,40	0.0001
	Chemical	8.93	4,40	0.0001	5.07	4,40	0.0021
	FxC	1.27	4,40	0.2964	3.2	4,40	0.0216

[a] Values expressed as arcsine √proportion.

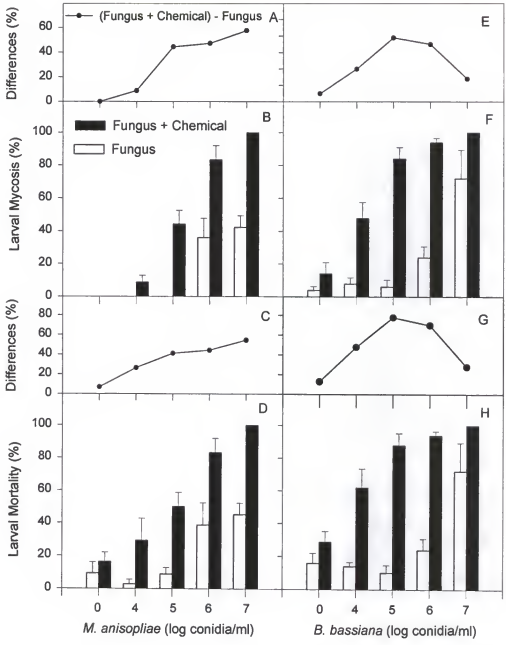

Fig. 5.1 Effect of different conidial concentrations of *Metarhizium anisopliae* (Test 1) and *Beauveria bassiana* (Test 2) applied to the cuticle alone (white bars) or in combination with a sublethal dose of imidacloprid at 100 ppm a.i. (black bars) on larval mortality (D, H) and mycosis (B, F) of first instar larvae of *Diaprepes abbreviatus* after 6 days. Synergism is expressed as the differences between the chemical with fungus and *M. anisopliae* or *B. bassiana* alone (Solid line, black dots) for larval mortality (C, G) and mycosis (A, E).

Mortality to first instar larvae of *D. abbreviatus* by *M. anisopliae* and *B. bassiana* with imidacloprid at 100 ppm ranged from 1.7-10.5 and 1.4-8.6 fold higher than for the fungi alone, respectively. Larval mortality and larval mycosis increased with an increase in conidial concentration of both *M. anisopliae* and *B. bassiana.* At lower conidial concentrations (10^4-10^5 conidia/ml), mycosis by both fungi was evident and exceeded 80% at the highest conidial concentrations (Fig. 5.1-B, F).

According to a probit analysis of the bioassay data (Table 5.2), the time required to kill 50% of the larvae (LT50), decreased significantly with the inclusion of imidacloprid in the conidial inoculum of both *M. anisopliae* and *B. bassiana*. The percent mortality and LT50 values for the *B. bassiana* compared to *M. anisopliae* with and without the chemical were consistently lower.

Effect of lower sublethal doses of imidacloprid (Test 3). Only small differences in larval mortality were detected between the different concentrations of imidacloprid by contact exposure alone (Fig. 5.2-D). For the chemical/fungus combination however, both larval mortality and larval mycosis increased with an increase in imidacloprid concentration (Fig. 5.2-B, D).

According to a factorial analysis of test 3 data, imidacloprid, *B. bassiana,* and the interaction had a significant effect on larval mortality (Table 5.1). Synergism was observed when concentrations of imidacloprid were 100 ppm or greater (Fig. 5.2-D). At concentrations below 100 ppm, differences in larval mortality were less than 20% between the chemical alone and the combination, with an additive effect (Fig. 5.2-C).

Table 5.2. Probit analysis of bioassay data where different conidial concentrations of *Metarhizium anisopliae* (test1) and *Beauveria bassiana* (test 2) with and without a sublethal dose of imidacloprid were tested against first instar larvae of *Diaprepes abbreviatus*.

Imidacloprid (ppm)	Concentration (Conidia/ml)	*M. anisopliae* LT50[c] (95% CI) (days)	*B. bassiana* LT50[c] (95% CI) (days)
0	0	16.0 (∞)	10.5 (7.7-34.7)
	10^4	16.6 (∞)	9.0 (7.2-16.5)
	10^5	12.9 (∞)	9.1(7.3-21.5)
	10^6	5.9 (4.6-18.1)	7.6 (6.5-10.5)
	10^7	5.5 (4.5-9.0)	3.9 (2.3-5.4)
100	0	9.3 (7.1-19.2)	7.4 (6.2-11.1)
	10^4	8.0 (6.2-15.7)	5.1(4.2-7.2)
	10^5	5.6 (5.1-6.4)	3.7 (2.4-4.7)
	10^6	3.9 (2.7-5.0)	2.1(1.8-2.5)
	10^7	2.5 (2.3-2.7)	2.0 (1.7-2.3)

[a] Means of 5 or 6 replicates for *B. bassiana* (n=50) and *M. anisopliae* (n=36), respectively.
[c] Time until death of 50% of inoculated larvae.

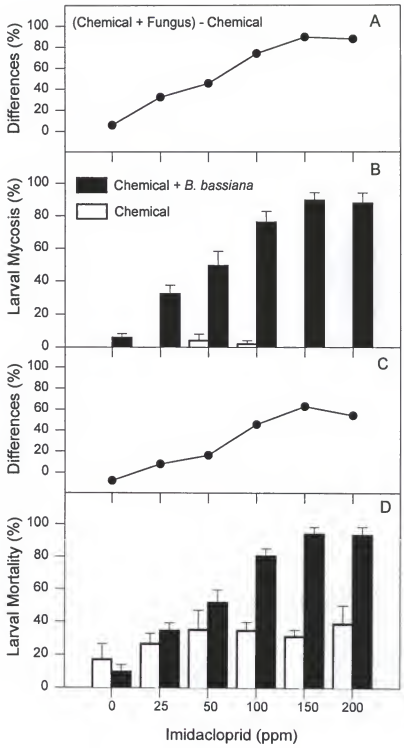

Fig. 5.2. Effect of different doses of imidacloprid applied to the cuticle alone (white bars) or in combination with *Beauveria bassiana* at 10^6 conidia/ml (black bars) on mortality (D) and mycosis (C) of first instar larvae of *Diaprepes abbreviatus* after 6 days (Test 3). Synergism is expressed as the differences between chemical with *Beauveria bassiana* and imidacloprid alone (Solid line, black dots) for larval mortality (C) and mycosis (A).

Larval mycosis was significant for both chemical and fungus, and the interaction was highly significant (Table 5.1). Some fungal infection was detected at 50 and 100 ppm in the chemical treatment (Fig. 5.2-B). The synergistic effect of the fungus/chemical combination, expressed as the difference in mortality or mycosis between imidacloprid alone and the combination, increased with an increase in conidial concentration (Fig. 5.2-A, C).

Probit analysis for the bioassay results are presented in Table 5.3. The LT50s for imidacloprid at 100, 150, and 200 ppm combined with the fungus at 10^6 conidia/ml, were significantly lower compared to chemical alone. For imidacloprid at doses below 100 ppm, the 95% CI for the LT50 values overlapped compared to chemical/fungus combination, suggesting that doses of imidacloprid less than 100 ppm do not synergize *B. bassiana.*

Effect of higher doses of imidacloprid. In test 4, only 20-40% increases in larval mortality were detected between the different concentrations of imidacloprid alone (Fig. 5. 3-D). Similarly, slight increases in larval mortality and mycosis occurred as the concentration of chemical/fungus increased (Fig. 5.3-B, D).

The factorial analysis for larval mortality was highly significant for chemical and fungus alone (Table 5.1). However, the chemical/fungal interaction was not significant, suggesting an additive effect. The additive effect expressed as the difference in larval mortality between imidacloprid alone and the chemical/fungal interaction, did not increase with an increase in the chemical concentration (Fig. 5.3-C).

Larval mycosis was significant for imidacloprid, *M. anisopliae* and the chemical/fungus interaction (Table 5.1). No larval mycosis was expected from imidacloprid alone, yet some was detected at 100, 500, and 1000 ppm from fortuitous contamination (Fig.

Table 5.3. Probit analysis of mortality data where different concentrations of imidacloprid with and without conidia of *Beauveria bassiana* were tested against first instar larvae of *Diaprepes abbreviatus* (test 3).

Concentration (conidia/ml)	Imidacloprid (ppm)	LT50 (95% CI) (days)
0	0	16.5 (∞)
	25	10.7 (7.3-59.7)
	50	6.7 (5.8-8.3)
	100	7.0 (6.0-9.3)
	150	7.1 (6.1-9.7)
	200	6.6 (5.7-8.5)
10^6	0	14.8 (∞)
	25	6.4 (4.9-20.4)
	50	5.1(3.9-11.1)
	100	3.9 (2.6-5.3)
	150	3.3 (2.7-3.8)
	200	3.7 (3.1-4.3)

[a] Means of five replicates (n=50).

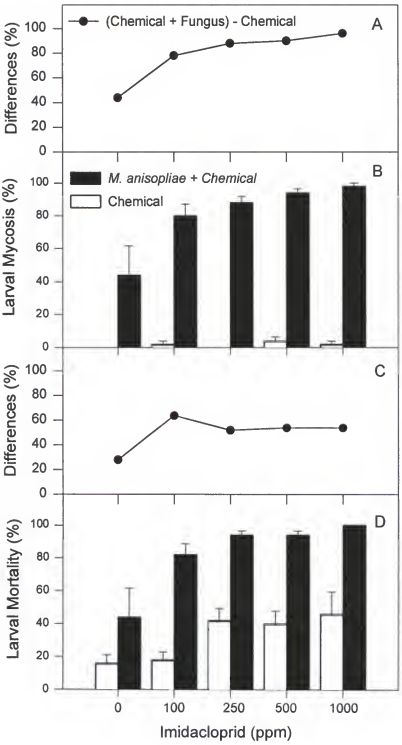

Fig. 5.3. Effect of different doses of imidacloprid applied to the cuticle alone (white bars) or in combination with *Metarhizium anisopliae* at 10^7 conidia/ml (black bars) on mortality (D) and mycosis (B) of first instar larvae of *Diaprepes abbreviatus* after 6 days (Test 4). Percent differences in larval mortality (C) and mycosis (A) between the combination with *M. anisopliae* and imidacloprid alone (Solid line, black dots).

5.3-B). The synergistic effect of the fungus/chemical interaction, increased with an increase in chemical concentration (Fig. 5.3-A).

Larval mortality was low for all doses of the fungus applied alone to the insect cuticle in test 5 (Fig. 5.4-B). Even the highest dose of the fungus at 10^7 conidia/ml killed only 18.6% of larvae after eight days. As the concentration of imidacloprid increased from 0 to 1000 ppm, mortality increased from 16.9 to 57.3%, respectively. However, when these same concentrations of imidacloprid were combined with the fungus at 10^5 conidia/ml, larval mortality increased significantly. For example, larval mortality was 12.7% at 0 ppm, 31.4% at 100 ppm, 58.4% at 500 ppm, and 80.9% at 1000 ppm of chemical. When imidacloprid at 500 and 1000 ppm was combined with the fungus at 10^6 and 10^7 conidia/ml, however, larval mortality did not increase. At 10^6 conidia/ml, larval mortality was higher when combined with 500 ppm (96%) than 1000 ppm (78%). In addition, there was no difference in larval mortality when imidacloprid at 500 or 1000 ppm was combined with the fungus at 10^7 conidia/ml.

The factorial analysis for larval mortality and mycosis in test 5 was significant for *B. bassiana* and for imidacloprid (Table 5.4). The *B. bassiana*-imidacloprid interaction was also significant. The synergistic effect of the fungus/chemical interaction increased with an increase in fungal conidial concentration (Fig. 5.4-C, D).

Larval mycosis was low for all fungus doses alone (Fig. 5.4-A). However, when *B. bassiana* at 10^6 and 10^7 conidia/ml was combined with imidacloprid at 500 ppm, the percent infection was 86 and 96%, respectively. When the same conidial concentration of the fungus were combined with imidacloprid at 1000 ppm, mycosis were reduced to 62.0 and

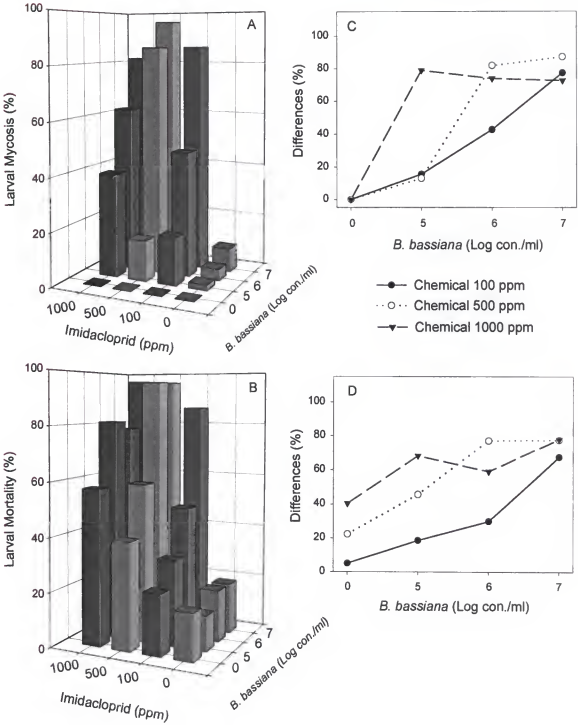

Fig. 5.4. Effect of different doses of imidacloprid applied to the cuticle alone (white bars) or in combination with different conidial concentrations of *Beauveria bassiana* on mortality (B) and mycosis (A) of first instar larvae of *Diaprepes abbreviatus* after 8 days (Test 5). Synergism is expressed as the difference between the fungus/chemical combination and *B. bassiana* alone for larval mortality (D) and mycosis (C).

Table 5.4. Summary of factorial analysis for larval mortality, mycosis and ecdysis of *Diaprepes abbreviatus* after treatment with *Beauveria bassiana* and imidacloprid alone and in combination (test 5).

Factor	Larval mortality[a]			Larval Mycosis[a]			Larval Ecdysis		
	F	df	P value	F	df	P value	F	df	P value
Fungus (F)	15.69	3,64	0.0001	90.41	3,64	0.0001	4.59	3,64	0.0057
Chemical (C)	36.16	3,64	0.0001	38.68	3,64	0.0001	131.49	3,64	0.0000
FxC	3.1	9,64	0.0001	8.71	9,64	0.0001	1.77	3,64	0.0923

[a] arcsine √proportion

81%, respectively.

The results of the probit analysis are presented in Table 5.5. The LT50 for the *Beauveria* treatments alone was significantly different only at 10^7 conidia/ml compared to *Beauveria*/imidacloprid combination at 100 ppm . Nevertheless, at 500 and 1000 ppm, all LT50 values for the treatment combinations were significantly different from the single treatments. The 95% CI for the LT50 values overlapped for the fungus/chemical combination at 500 ppm and at 1000 ppm.

As concentrations of imidacloprid increased, larval ecdysis decreased in all treatments (Fig. 5.5). Factorial analysis for larval ecdysis was highly significant for imidacloprid, since virtually no larval ecdysis was observed at 500 ppm and 1000 ppm (Table 5.4). However, the fungus alone had minimal effect on larval ecdysis. Only an additive effect for larval ecdysis was observed since the interaction was not significant (Table 5.4).

Comparison between oral and cuticular exposure of imidacloprid. In test 5, mortality and mycosis among larvae treated with *M. anisopliae* alone, for both exposure methods, was less than 20% (Fig. 5.6-D, H). In fact, no larval mycosis caused by *M. anisopliae* was observed at 10^5 and 10^6 conidia/ml for contact and oral exposure, respectively. However, when *M. anisopliae* conidia were combined with imidacloprid, a gradual increase in larval mortality and mycosis occurred with an increase in fungal concentration for both contact and oral exposure (Fig. 5.6-D, H).

The factorial analysis for larval mortality and mycosis was significant for both imidacloprid and *M. anisopliae* using both contact and oral exposure (Table 5.6). The

Table 5.5. Probit analysis of mortality data where different dosages of imidacloprid with and without different conidial concentrations of *Beauveria bassiana* were tested against first instar larvae of *Diaprepes abbreviatus* (test 5).

Imidacloprid (ppm)	Concentration (conidia/ml)	LT50[c] (95% CI) (days)
0	0	14.2 (10.0-730.1)
	10^5	11.1 (9.1-41.0)
	10^6	10.6 (8.9-19.1)
	10^7	12.2(9.5-38.3)
100	0	15.7 (∞)
	10^5	9.8 (8.3-14.2)
	10^6	7.8 (6.8-9.6)
	10^7	3.6 (1.8-4.5)
500	0	9.5 (7.9-15.5)
	10^5	7.2 (6.4-8.4)
	10^6	4.9 (4.5-5.4)
	10^7	4.1 (3.4-4.6)
1000	0	7.2 (6.1-9.4)
	10^5	6.4 (5.8-7.0)
	10^6	5.2 (4.5-5.9)
	10^7	3.5 (2.7-4.0)

[a] Means of five replicates (n=50).

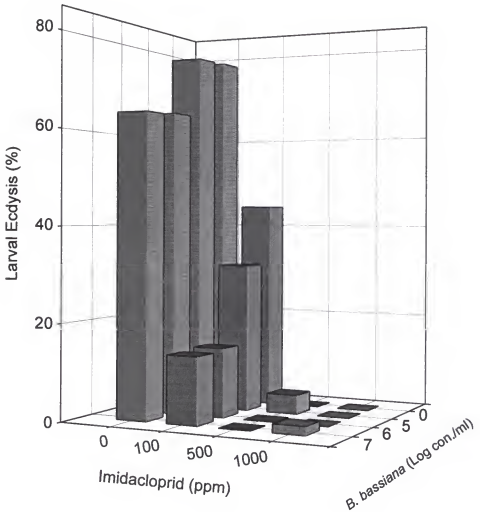

Fig. 5.5. Effect of different doses of imidacloprid applied directly to the cuticle of first instar larvae of *Diaprepes abbreviatus* alone or in combination with different conidial concentrations of *Beauveria bassiana* on larval ecdysis (Test 5).

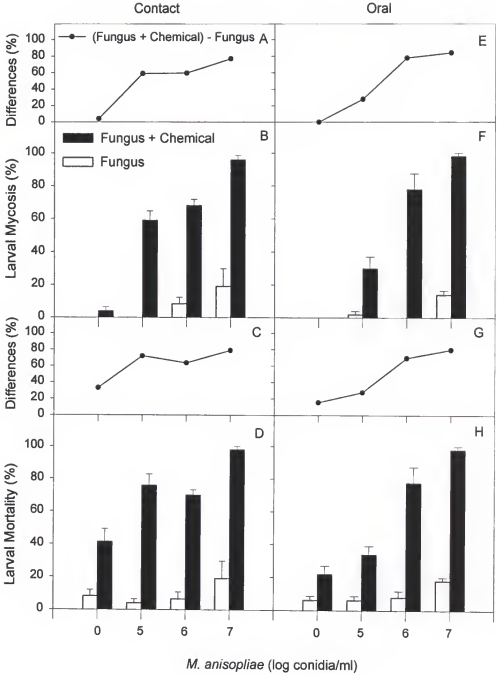

Fig. 5.6. Effect of different conidial concentration of *Metarhizium anisopliae* applied directly to the cuticle alone (white bars) or in combination with imidacloprid applied as contact (A-D) or oral (E-H) treatment at 100 ppm a.i. (black bars) on mortality and mycosis of first instar larvae of *Diaprepes abbreviatus* after 6 days (Test 6). Percent differences in larval mortality (C, G) and mycosis (A, E) between the fungus/imidacloprid interaction and *M. Anisopliae* alone for contact and oral exposure (Solid line, black dots).

Table 5.6. Summary of factorial analysis for larval mortality, mycosis, and ecdysis of *Diaprepes abbreviatus* following treatment with conidia of *Metarhizium anisopliae* alone or in combination with imidacloprid applied as a contact or oral treatment.

Exposure	Factor	Larval mortality[a]			Larval Mycosis[a]			Larval Ecdysis		
		F	df	P value	F	df	P value	F	df	P value
Contact	Fungus (F)	11.26	3,32	0.0001	34.57	3,32	0.0001	3.47	3,32	0.0274
	Chemical (C)	223.63	1,32	0.0001	128.5	1,32	0.0001	56.84	1,32	0.0001
	FxC	5.95	3,32	0.0024	10.35	3,32	0.0001	0.98	3,32	0.4162
Oral	Fungus (F)	37.31	3,32	0.0001	95.61	3,32	0.0001	0.85	3,32	0.4760
	Chemical (C)	210.34	1,32	0.0001	286.31	1,32	0.0001	228.98	1,32	0.0001
	FxC	22.10	3,32	0.0001	46.04	3,32	0.0001	0.85	3,32	0.4760

[a] Values expressed as arcsine √proportion.

fungus/chemical interaction was also significant confirming synergism (Table 5.6). The synergistic effect of the fungus/chemical interaction on contact and oral exposure, increased with an increase in fungal conidial concentration (Fig. 5.6-A, C, E, G).

Factorial analysis for larval ecdysis showed that contact-treated larvae were affected by imidacloprid and M. anisopliae but the interaction was not significant, suggesting an additive effect (Table 5.6). Larval ecdysis was 25.6% for imidacloprid at 100 ppm, but when combined with the fungus at 105 and 107 conidia/ml, ecdysis droped to 4% and 0% respectively (Fig. 5.7 B). Oral treatment of larvae with imidacloprid had a greater effect on larval development (Fig 5.7-A). No larval ecdysis was observed for chemical alone or in combination with the fungus (Table 5.6). However, the fungus alone had no effect on larval ecdysis and the fungus/imidacloprid interaction was not significant.

A probit analysis of the bioassay results is presented in Table 5.7. The time required to kill 50% of the larvae decreased with an increase in conidial concentration of *M. anisopliae* alone or in combination with chemical for oral exposure. For both exposure methods, the LT50 values for the chemical/fungus treatment was significantly lower than that for fungus alone. The LT50 for imidacloprid at 100 ppm with fungus at 10^5 conidia/ml on contact exposure was significantly lower than for the oral treatment at this same dosage of fungus/chemical combination. For the other treatments, the 95% CI for the LT50 values overlapped for oral and contact exposures.

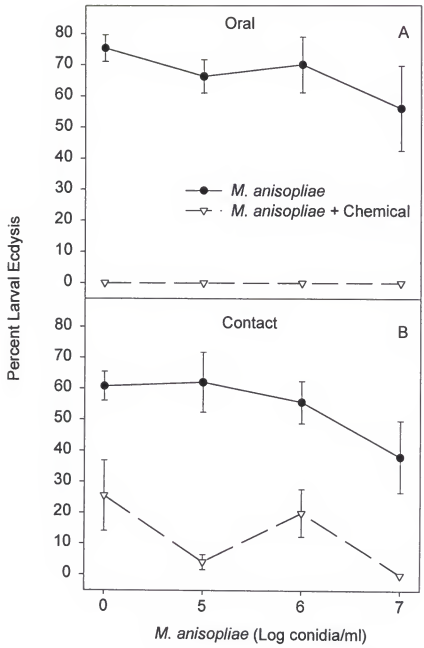

Fig. 5.7. Effect of different conidial concentrations of *Metarhizium anisopliae* applied alone (solid line, black dots) to the cuticle of first instar of *Diaprepes abbreviatus* (white bars) or with imidacloprid (dashed line , white dots) applied via contact (B) and oral (A) exposure on ecdysis (Test 6).

Table 5.7. Probit analysis of bioassay data where different conidial concentrations of *Metarhizium anisopliae* with and without a sublethal dose of imidacloprid were applied via contact and oral exposure against first instar larvae of *Diaprepes abbreviatus* (test 6) .

Imidacloprid (ppm)	Concentration (Conidia/ml)	Contact LT50[c] (95% CI) (days)	Oral LT50[c] (95% CI) (days)
0	0	17.9 (11.1-2756.5)	14.9 (10.3-59.2)
	10^5	8.8 (7.7-18.3)	10.2 (8.4-16.8)
	10^6	11.1 (8.9-19.3)	9.6 (8.2-14.5)
	10^7	9.2 (7.7-12.9)	9.5 (8.0-13.1)
100	0	6.9 (6.1-8.3)	9.7 (7.9-14.2)
	10^5	4.4 (3.5-5.3)	7.8 (6.8-9.7)
	10^6	4.0 (1.5-6.4)	4.0(3.6-4.3)
	10^7	2.0 (1.8-2.2)	2.4 (1.2-3.1)

[a] Means of five replicates (n=50).
[c] Time until death of 50% of inoculated larvae.

Cuticular Exposure to Formulated Imidacloprid, and Each Component Separately with Conidia of *M. Anisopliae*.

The active ingredient and the formulated product combined with fungal conidia had a significantly greater effect on larval mortality and mycosis than each component alone (Table 5.8). The LT50 values for these treatments were also reduced. The inert carrier alone had no effect on larval mortality compared to the control. When combined with *M. anisopliae* conidia, the inert carrier killed 10% more larvae than the fungus alone, but the difference was not significant. These results suggest that the active ingredient is the key component of the formulation responsible for the synergism between imidacloprid and the entomopathogenic fungi.

Table 5.8. Probit analysis of bioassay data where inert carrier (IC) , active ingredient(AI), and formulated imidacloprid (AI+IC) with and without conidia of *Metarhizium anisopliae* were tested against first instar larvae of *Diaprepes abbreviatus.*

Dose (conidia/ml)	Chemical ingredient[b]	LT50[d] (95% CI) (days)	% Mortality ± SE[e]	% mycosis ± SE[e]
0	0	11.3 (8.6-8,562.4)	5.6 ± 3.7 c	0 c
	IC	22.2 (∞)	6.2 ± 4.1bc	0 c
	AI	8.5 (7.3-11.3)	30.4 ± 6.7 b	6.2 ± 4.1bc
	AI+IC	11.0 (8.1-33.2)	29.4 ± 8.4bc	0 c
10^7	0	11.5 (8.8-25.4)	16.5 ± 2.3bc	8.7 ± 2.2bc
	IC	8.7 (7.5-11.8)	26.7 ±5.3bc	14.4 ± 4.2b
	AI	4.3 (2.8-5.4)	87.7 ± 4.0a	70.8 ± 5.6a
	AI +IC	3.8 (2.2-4.9)	91.8 ± 2.1a	83.5 ± 2.7a

[a] Means of five replicates (n=50).
[b] Active ingredient at 500 ppm and inert carrier at 1,836 ppm.
[c] Means followed by the same letter are not significantly different by Tukey's test (P=0.05).

CHAPTER 6
SYNERGISTIC EFFECT OF TWO ENTOMOPATHOGENIC FUNGI AND IMIDACLOPRID ON THE BEHAVIOR AND SURVIVAL OF LARVAE OF *Diaprepes abbreviatus* (COLEOPTERA: CURCULIONIDAE) IN SOIL.

Introduction

In chapter 3, oral and cuticular treatment of first instar larvae with *M. anisopliae* or *B. bassiana* in combination with sublethal doses of imidacloprid synergistically increased larval mortality and mycosis of *D. abbreviatus* in a non-soil substrate. The purpose of this chapter was to determine whether the fungal/chemical combination performs similarly in soil. Different concentrations and formulations of *M. anisopliae* and *B. bassiana* with and without imidacloprid were tested to determine their effect on mobility, mortality, and mycosis of first instar larvae of *D. abbreviatus.* In addition, the fungus/imidacloprid interaction was studied in soil with different moistures.

Materials and Methods

General procedures relating to insect rearing, chemicals, and soil type have been previously described in chapter 2.

Metarhizium anisopliae (strain MADA) was isolated from *D. abbreviatus* larvae collected from citrus grove soil at Apopka, FL. A strain of *Beauveria bassiana*, isolated from a grasshopper, and commercialized as Mycotrol by Mycotech Corporation was used in the experiments. Two conidial formulations of *B. bassiana*, an oil suspension and a wettable powder (WP) were used in our experiments. Conidia of *M. anisopliae* were

produced in the manner described in chapter 3.

Larval Mobility in Soil at Different Moistures.

Two preliminary experiments were conducted to determine the effect of larval age on vertical movement in Candler sandy soil. In test one, newly-hatched and 48-h-old larvae were exposed to soil at different moisture levels. Four g of soil were moistened with sterilized distilled water to 0, 2, 4, 6, 8, 10, 12 % water content (v/w) and added to a depth of 2 cm to bioassay columns (15 cm ht, 2.0 cm diam.) constructed from polystyrene tubes similar to those described by Hamlen and Beavers (1975). Twenty newly hatched or 48-h-old first instar larvae were placed on the soil surface of each container. Bioassay units were closed tightly and held at 28 °C. Each treatment combination was replicated four times. After 24 and 48 h, the number of insects recovered in a containment cell at the base of the bioassay column was recorded microscopically at 16x.

In test 2, soil depth at the same soil moistures were tested to determine its effect on larval movement. Two, 4, 6, and 8 g of Candler soil moistened to 0, 2, 6, 8, 10, and 12% water content (v/w) were added to a depth of 1, 2, 3, and 4 cm to bioassay columns, respectively. Twenty 48-h-old first instar larvae were placed in the plastic column in the manner described previously. Each treatment was replicated 4 times.

Larval Mobility, Mortality, and Mycosis Following Soil Treatment with *B. bassiana*, *M. anisopliae*, and Imidacloprid.

Soil Treatment with Mycotrol Wettable Powder. Four ml of imidacloprid at concentrations of 0, 100, 500, and 1000 ppm and Mycotrol WP at 10^5, 10^6, 10^7 conidia/ml were added alone or in combination to 50 g of Candler soil. These dosages were equivalent

to imidacloprid at 0, 8, 40, and 80 μg a.i./g soil and Mycotrol at 0, $8x10^3$, $8x10^4$, and $8x10^5$ conidia/g soil, respectively. The soil was moistened to 8% water content (v/w) when adding the treatments. After mixing thoroughly, 2 g of treated soil were added to a depth of 1 cm to each bioassay columns (15.0 cm h x 2.0 cm diam.) containing 2 g of untreated soil at 8% water content. Bioassay columns were then attached to a containment unit used to catch the invasive larvae. Each column cell within the unit contained a 1.5 mm dia. moist filter paper and a piece of sliced carrot as a food source. Twenty 48-h-old first instar larvae were placed on the soil surface of each container. Bioassay units were closed tightly and held at 28° C. Each treatment was replicated 4 times. After 7 d, the number of live and dead larvae recovered from the containment cell as well as those remaining in the soil was recorded by microscopic examination. Dead larvae were held in 35 mm Petri dishes with 1.5 mm dia. moistened filter paper to confirm mycosis by *B. bassiana.*

Soil Treatment with Mycotrol Formulated in Oil and Conidia of *M. anisopliae.* One and half ml of imidacloprid at concentrations of 0, 100, 500, and 1000 ppm and *B. bassiana* as Mycotrol formulated in oil and *M. anisopliae* at 10^5, 10^6, 10^7 conidia/ml were mixed alone or in combination into 30 g of Candler soil. These concentrations were equivalent to imidacloprid at 0, 5, 25, and 50 μg ai/g soil and fungus at 0, $5x10^3$, $5x10^4$, and $5x10^5$ conidia/g soil, respectively. Soil was moistened to 5% water content (v/w) when adding the treatments. Two g of treated soil were added to a depth of 1 cm to each bioassay column. Ten 48-h-old larvae were added to each column. Each treatment was replicated 5 times.

Soil Treatment with *M. anisopliae* and Imidacloprid at Different Soil Moistures. A volome of 1.5 ml of imidacloprid at concentrations of 0 and 500 ppm and *M. anisopliae* at 0 and 10^7 conidia/ml were added alone or in combination to 30 g of Candler soil. These concentrations were equivalent to imidacloprid at 0 and 50 μg a.i./g soil and *M. anisopliae* at 0 and $5x10^5$ conidia/g soil, respectively. The treated soil was moistened to 0, 7%, and 14% water content (v/w). At 0% moisture, the treated soil was air-dried in a dark incubator at 28^0 C with continuous air flow. Other experimental protocols were the same as described for the previous experiment.

Statistical analysis.

All bioassay data were transformed to arcsine $\sqrt{x}$ before performing regression and factorial analysis using the general linear model procedure. Correlation analysis between larval locomotory response in soil and larval mortality and mycosis were performed using the correlation procedure (SAS Institute 1985).

Results

Larval Mobility in Soil at Different Moistures.

In test 1, vertical movement of larvae through soil was only slightly greater after 48 h compared to 24 h (Fig. 6.1). At 0% moisture, less than 20% of the larvae were recovered and larval age was not a factor. Mobility was higher for 48-h-old compared to newly-hatched larvae at all soil moistures except that ranging from 0-4% where emergence was similar. The relationship between larval movement and soil moisture best fit a quadratic equation for 48-h-old larvae at both 24 (R^2= 0.66; F=29.22; df=2,25; P< 0.0001) and 48 h (R^2= 0.60; F=23.94; df=2,25; P< 0.0001). Regression analysis between movement of

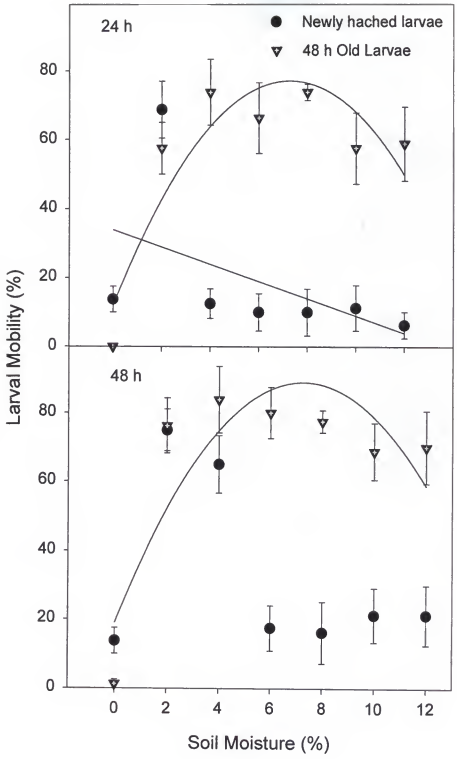

Fig. 6.1. Mobility of newly hatched and 48 h old larvae of *Diaprepes abbreviatus* in Candler sandy soil at different moistures.

newly-hatched larvae and soil moisture was poorly correlated with a linear model at 24 h (r^2= 0.23; F=7.66; df=1,26; P< 0.0105) and not significant at 48h (r^2= 0.15; F=3.55; df=1,26; P< 0.0711). It is difficult to explain why the vertical movement of new-hatched larvae was limited to a very narrow range of moistures (2-4%) and dropped off drastically at 6% in view of the fact that 48 h larvae appeared unaffected. Further research is needed to clarify these findings.

In test 2, the relationship of larval recovery to soil moisture and depth changed over time (Fig. 6.2). After 24 h post-inoculation, vertical movement of larvae increased as soil moisture and depth decreased, yet the combined effect of soil depth and moisture on larval mobility was not significant (Table 6.1). However, at 12% soil moisture there was a 2 fold-difference in larval movement between a depth of 1 cm and 2-4cm. After 72 h, most larvae completed their vertical descent at 2% moisture at all soil depths. At 6-12% soil moisture however, larval mobility decreased linearly with an increase in soil depth (Table 6.1). At 120 h, virtually all larvae successfully moved through 4 cm soil regardless of soil moisture and depth. The relationship between larval mobility, soil moisture, and depth best fit a quadratic curve at 120 h (Table 6.1). It appears that larval mobility at different soil moistures and depths was delayed in time only.

Larval Mobility, Mortality, and Mycosis Following Soil Treatment with *B. bassiana*, *M. anisopliae*, and Imidacloprid.

Soil Treatment with Mycotrol Wettable Powder. Different concentrations of conidia of *B. bassiana* as Mycotrol WP applied to the soil had no significant effect on larval mobility (Fig. 6.3-A, Table 4.2). However, all concentrations of imidacloprid alone or in combination

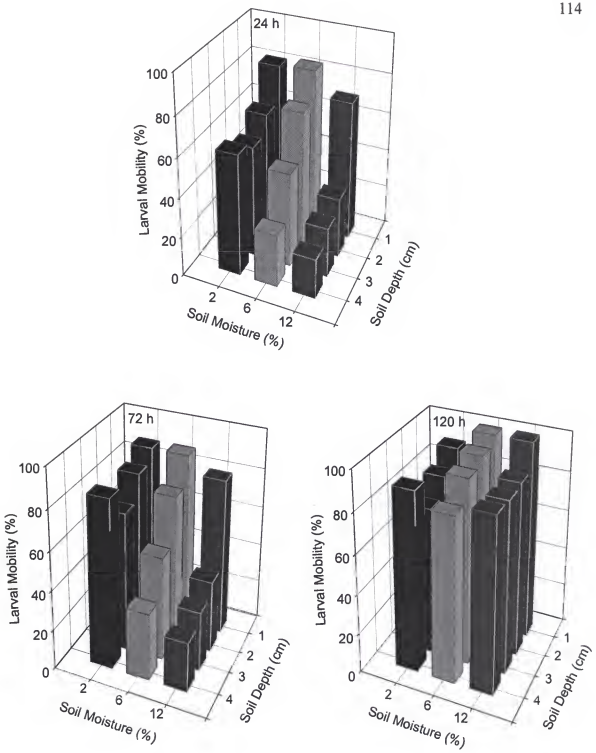

Fig. 6.2. Effect of different moisture contents and soil depth on larval mobility of first instar larvae of *Diaprepes abbreviatus* after 24, 72, and 120 h.

Table 6.1. Summary of factorial and regressions analysis for larval mobility of *Diaprepes abbreviatus* at different soil depths (D) and moistures (M) after 24, 72, and 120 h.

Factor	24 h [a]			72 h [a]			120 h [a]		
	F	df	P value	F	df	P value	F	df	P value
M	29.17	1,42	0.0001	56.81	1,42	0.0001	6.45	1,42	0.0150
MxM	1.59	2,41	0.2151	0.0	2,41	0.9488	5.28	2,41	0.0268
D	55.08	1,42	0.0001	47.78	1,42	0.0001	13.89	1,42	0.0006
DxD	4.01	2,41	0.0518	5.47	2,41	0.0242	7.28	2,41	0.0101
MxD	3.34	2,41	0.075	9.74	2,41	0.0033	2.95	2,41	0.0935

[a] The R-square for the model on data transformed to arcsine √proportion is 0.70, 0.75, and 0.49 for 24, 72, and 120 h, respectively.

with fungal conidia had a significant effect on larval mobility. Larval mobility decreased with an increase in concentration of imidacloprid and best fit a second degree curve (Table 6.2). The fungal/chemical interaction had no significant effect on larval mobility.

Larval mortality and mycosis at different conidial concentrations of Mycotrol ranged from 18-43% and 0-33%, respectively, with no apparent dose response (Fig. 6.4-A, B). Imidacloprid alone increased larval mortality, particularly at 8 and 80 μg/g of soil. The fungal/chemical interaction, however, significantly increased both larval mortality and mycosis (Table 6.2). For example, larval mortality at 8x 10^3 conidia/ml was 18%, but in combination with imidacloprid at doses ranging from 8 to 80 μg/g soil, mortality ranged from 87.4-98%. Larval mycosis was less than larval mortality for the fungus alone and the combination (Fig 6.4-B). In this bioassay, a 1cm layer of untreated soil was added to the bioassay columns before adding treated soil. Probably, most healthy larvae void their cuticle of conidia as they moved through untreated soil (See chapter 3). Larval mortality ($r=-0.89$, $P< 0.0001$) and larval mycosis ($r=-0.55$, $P<0.001$) in soil were negatively correlated with larval mobility in soil. Higher levels of larval mortality and mycosis were observed when fewer larvae moved vertically through Candler soil.

All treatments alone or in combination affected both larval mortality and mycosis significantly according to factorial analysis (Table 6.2). Synergism between the fungus and chemical was proven by the significance of the main effects, the interaction, and the increased differences in larval mortality or mycosis between fungus/chemical and fungus alone (Fig. 6.4-C, D).

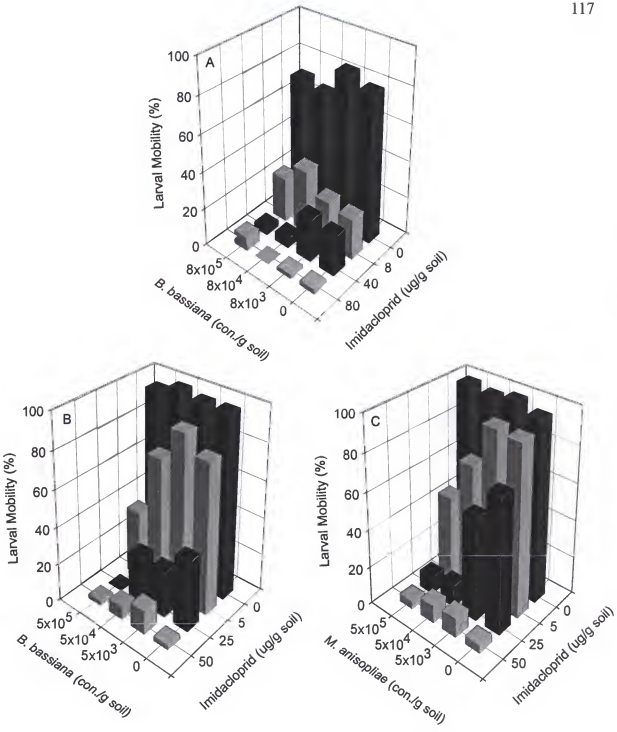

Fig. 6.3. Effect of different doses of imidacloprid applied alone to Candler sandy soil or in combination with different conidial concentrations *Beauveria bassiana* wettable powder (A), or *B. bassiana* oil formulation (B), or *M. anisopliae* (C) on the mobility of first instar larvae of *Diaprepes abbreviatus*. For *B. bassiana* wettable powder (A) the soil moisture was held constant at 8%. For *B. bassiana* oil formulation (B) and *M. anisopliae* (C) the soil moisture was 5%.

Table 6.2. Summary of factorial and regressions analysis for larval mobility in soil, larval mortality, and mycosis of *Diaprepes abbreviatus* subsequent to soil treatment with *Beauveria bassiana* (Bb) and imidacloprid (IMI) alone and in combination.

Test	Factor	Larval locomotory response (R^2=0.69)[a]			Larval mortality (R^2=0.71)[a]			Larval mycosis (R^2=0.78)[a]		
		F	df	P value	F	df	P value	F	df	P value
1	IMI	107.03	1,58	0.0001	76.64	1,58	0.0001	41.28	1,58	0.0001
	IMIxIMI	15.19	2,57	0.0003	11.74	2,57	0.0011	17.21	2,57	0.0001
	Bb	0.33	1,58	0.5669	34.69	1,58	0.0001	127.52	1,58	0.0001
	BbxBb	0.81	2,57	0.3732	1.15	2,57	0.2888	0.30	2,57	0.5849
	IMIxBb	0.00	2,57	0.9951	6.95	2,57	0.0108	8.31	2,57	0.0055
	IMIxIMIxBb	1.31	3,57	0.2563	8.39	3,57	0.0054	5.56	3,57	0.0218

[a] R-square for the model on data transformed to arcsine √proportion.

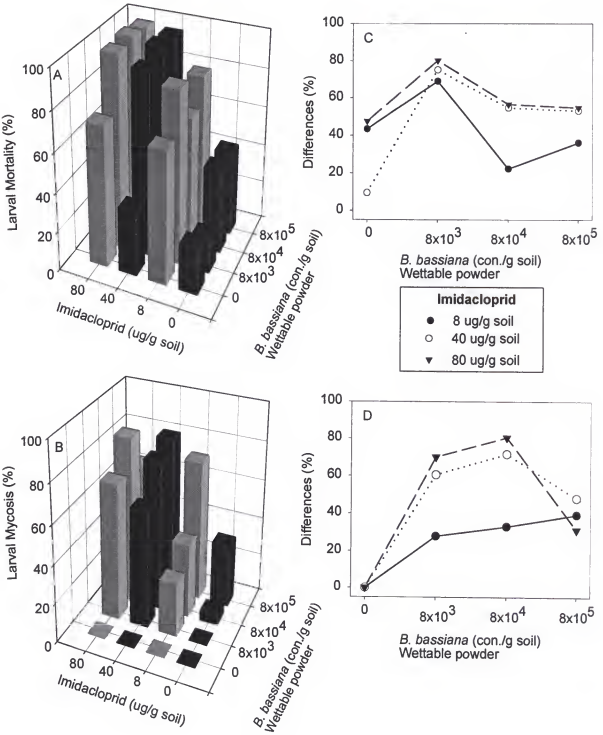

Fig. 6.4. Effect of different doses of imidacloprid applied alone to Candler soil or in combination with different conidial concentrations of *Beauveria bassiana* WP on mortality (A) and mycosis (B) of first instar larvae of *Diaprepes abbreviatus.* Synergism expressed as the differences between the combination chemical/fungus and *B. bassiana* alone for larval mortality (C) and mycosis (D).

Soil Treatment with *B. bassiana* Formulated in oil and Conidia of *M. anisopliae*. Conidia of *B. bassiana* formulated in oil as Mycotrol and conidia of *M. anisopliae* had no effect on mobility of 48-h-old larvae in Candler soil (Fig. 6.3-B, C). As was true in the previous test, larval mobility in soil decreased significantly with an increase in the concentration of imidacloprid. The effect of imidacloprid on larval movement was greater when combined with different concentrations of both *B. bassiana* or *M. anisopliae* and best fit a second degree equation for both fungi and chemical (Table 6.3, tests 1, 2). The factorial analysis for larval mobility was significant for *B. bassiana*, *M. anisopliae*, and imidacloprid alone (Table 6.3, tests 1, 2). Contrary to the previous test with Mycotrol WP, the Mycotrol in oil-imidacloprid and *M. anisopliae*-imidacloprid interactions had a greater effect on larval mobility than either treatment alone. Larval mortality caused by Mycotrol in oil and *M. anisopliae* alone was low ranging from 4.9-14.2% and 2.0-8.0%, respectively. Larval mycosis was observed only at $5x10^5$ conidia/g soil (10.2%) for Mycotrol and no mycosis was caused by *M. anisopliae* alone (Figs. 6.5, 6.6 B). However, when sublethal doses of imidacloprid were included with the fungi, both larval mortality and mycosis increased significantly (Figs. 6.5, 6.6 A, B). Even where no mycosis was recorded for the fungus alone, the addition of imidacloprid resulted in infection. For example, at 50 μg/g soil of imidacloprid, mycosis levels at $5x10^3$ and $5x10^0$ conidia/ml were 59.5 and 75.4%, respectively. This suggests that imidacloprid influenced the infection process. Larval mortality ($r=-0.87$ $p<0.0001$) and mycosis ($r=-0.72$, $p<0.0001$) for *B. bassiana* was highly correlated with larval mobility in soil. Similarly for *M. anisopliae*, larval mortality ($r=-0.85$, $p<0.0001$) and mycosis ($r=-0.77$, $P<0.0001$) were negatively correlated with larval

Table 6.3. Summary of factorial and regressions analysis for larval mobility in soil, larval mortality, and mycosis of *Diaprepes abbreviatus* subsequent to soil treatment with *Beauveria bassiana* (Bb) (test 1) , *Metarhizium anisopliae* (Ma) (test 2), and imidacloprid (IMI) alone and in combination.

Test	Factor	Larval locomotory response [a]			Larval mortality [a]			Larval mycosis [a]		
		F	df	P value	F	df	P value	F	df	P value
1	IMI	368.70	1,74	0.0001	123.06	1,74	0.0001	207.58	1,74	0.0001
	IMIxIMI	47.70	2,73	0.0001	9.22	2,73	0.0033	16.51	2,73	0.0001
	Bb	7.61	1,74	0.0073	44.86	1,74	0.0001	206.84	1,74	0.0001
	BbxBb	20.45	2,73	0.0001	23.06	2,73	0.0001	44.12	2,73	0.0001
	IMIxBb	1.43	2,73	0.2358	4.95	2,73	0.0291	68.19	2,73	0.0001
	IMIxIMIxBb	6.84	3,73	0.0108	0.49	3,73	0.4846	11.74	3,73	0.0010
2	IMI	307.92	1,74	0.0001	112.84	1,74	0.0001	144.55	1,74	0.0001
	IMIxIMI	12.95	2,73	0.0006	3.62	2,73	0.0609	14.22	2,73	0.0003
	Ma	15.70	1,74	0.0002	84.70	1,74	0.0001	169.35	1,74	0.0001
	MaxMa	8.09	2,73	0.0058	25.37	2,73	0.0001	19.45	2,73	0.0001
	IMIxMa	0.60	2,73	0.4405	42.86	2,73	0.0001	52.58	2,73	0.0001
	IMIxIMIxMa	25.84	3,73	0.0001	25.85	3,73	0.0001	9.70	3,73	0.0026

[a] In test 1, the R-square for the model is 0.86, 0.73, and 0.88 for larval locomotory response, larval mortality, and larval mycosis, respectively. In test 2, the R-square for the model is 0.83, 0.80, and 0.85 for larval locomotory response, larval mortality, and larval mycosis, respectively. In both test, data were transformed to arcsine √proportion before analysis.

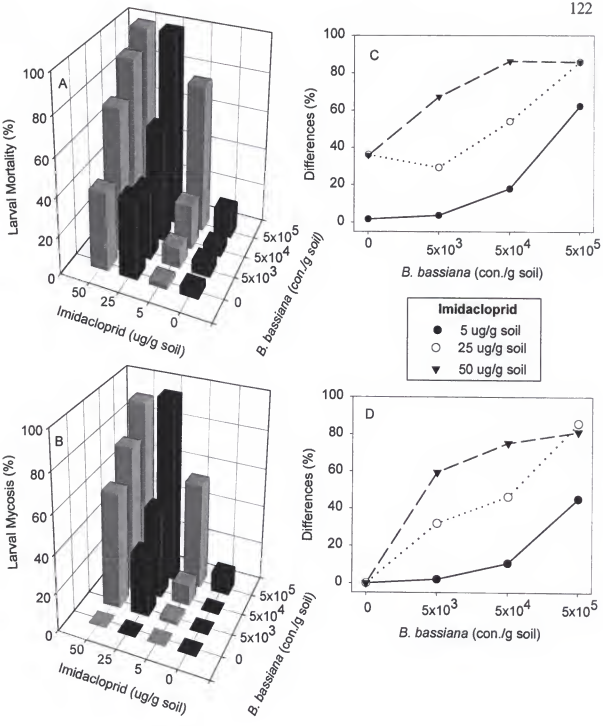

Fig. 6.5. Effect of different doses of imidacloprid applied alone to Candler soil or in combination with different conidial concentrations of *Beauveria bassiana* oil formulation on mortality (A) and mycosis (B) of first instar larvae of *Diaprepes abbreviatus.* Synergism expressed as the differences between the combination chemical/fungus and *B. bassiana* alone for larval mortality (C) and mycosis (D).

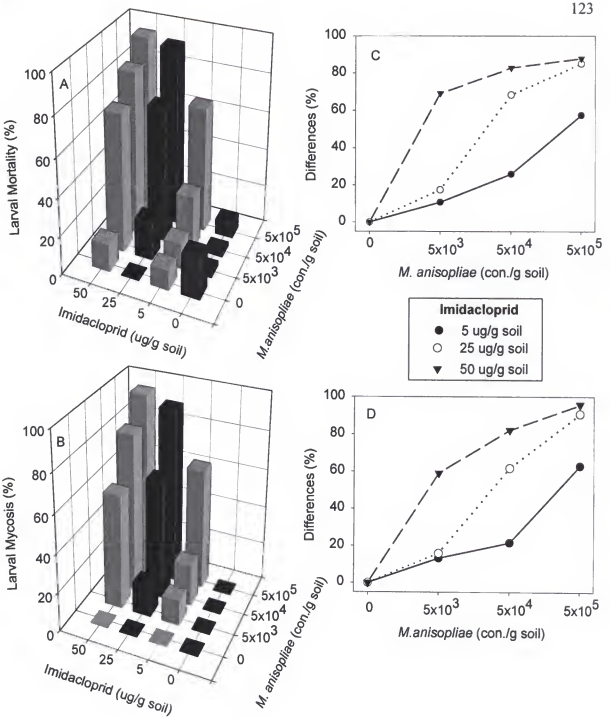

Fig. 6.6. Effect of different doses of imidacloprid applied alone to Candler soil or in combination with different conidial concentrations of *Metarhizium anisopliae* on mortality (A) and mycosis (B) of first instar larvae of *Diaprepes abbreviatus.* Synergism expressed as the differences between the combination chemical/fungus and *M. anisopliae* alone for larval mortality (C) and mycosis (D).

movement. Mortality and mycosis were highest when larval mobility in soil was lowest.

The factorial analysis for larval mortality and mycosis was significant for *B. bassiana*, *M. anisopliae*, and for imidacloprid (Table 6.3, tests 1, 2). The *B. bassiana*-imidacloprid and *M. anisopliae*-imidacloprid interactions were also significant. The synergistic effect of the fungus/chemical combination, expressed as the difference in mortality or mycosis between the combination and *M. anisopliae* or *B. bassiana* alone increased with an increase in fungal conidial concentration (Figs. 6.5, 6.6 C, D). In addition, differences in mortality and mycosis for *B. bassiana* and *M. anisopliae* experiments increased as doses of imidacloprid increased from 5 to 50 μg/soil.

Soil treatment with *M. anisopliae* at different soil moisture. Similar to the previous test, *M. anisopliae* alone at $5x10^5$ conidia/g had no effect on larval movement, but imidacloprid at 25 μg/g soil significantly impaired larval movement in soil moistures ranging from 0-14% (Fig. 6.7-A, Table 6.4). As was the case in preliminary studies, 0% soil moisture masked treatment effects by inhibiting larval mobility. Only 20% of the surviving larvae moved through the soil column, while 38% remained alive in dry soil after 7 days.

In the case of larval mortality, 42% of the larvae died in dry soil while higher moistures had no effect. The addition of imidacloprid alone had virtually no effect on larval mortality. However, *M. anisopliae* killed 82.4, 12.5, and 35% of larvae at 0, 7, and 14% soil moisture, respectively. Interestingly, all the cadavers infected with *M. anisopliae* failed to sporulate in dry soil. However, sporulation was stimulated by exposure to moist conditions. Mycosis occurred at 0 and 14% soil moistures but not at 7% when only fungus was included in the soil. In the fungus/chemical treatment, both larval mortality and mycosis decreased

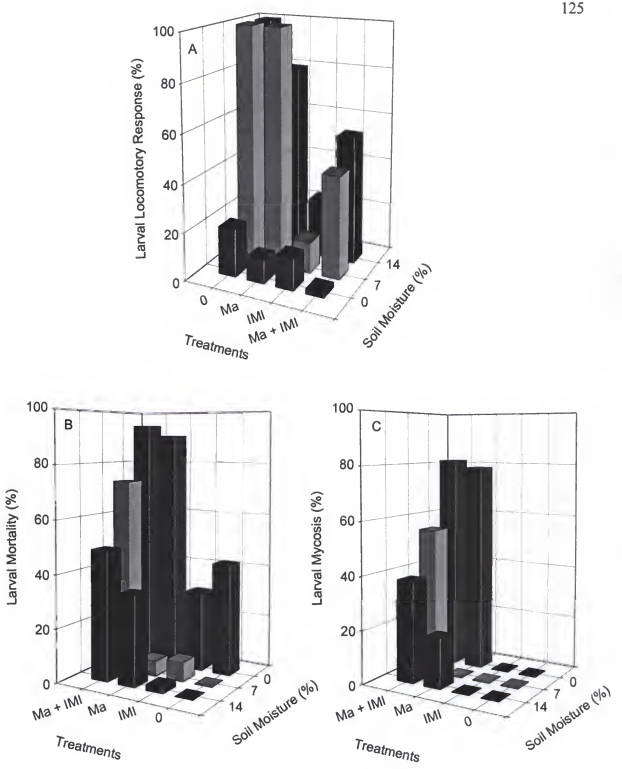

Fig. 6.7. Effect of imidacloprid applied alone to Candler soil or in combination with conidial concentrations of *Metarhizium anisopliae* on larval mobility (A), mortality (B) and mycosis (C) of first instar larvae of *Diaprepes abbreviatus* under different soil moisture.

Table 6.4. Summary of factorial and regressions analysis for larval mobility in soil, larval mortality, and mycosis of *Diaprepes abbreviatus* subsequent to soil treatment with *M. anisopliae* (Ma) and imidacloprid (IMI) alone and in combination under different soil moisture (Moist).

Factor	Larval locomotory response (R^2=0.77)[a]			Larval mortality (R^2=0.80)[a]			Larval mycosis (R^2=0.78)[a]		
	F	df	P value	F	df	P value	F	df	P value
IMI	66.36	1,52	0.0001	9.51	1,52	0.0032	9.09	1,52	0.0040
Ma	0.32	1,52	0.5730	95.52	1,52	0.0001	111.81	1,52	0.0001
Moist	65.97	1,52	0.0001	60.85	1,52	0.0001	24.35	1,52	0.0001
MoistxMoist	20.93	2,51	0.0001	19.64	2,51	0.0001	8.56	2,51	0.0051
IMIxMa	5.95	2,51	0.0182	10.83	2,51	0.0018	9.09	2,51	0.0040
IMIxMoist	11.19	2,51	0.0016	1.31	2,51	0.2576	0.67	2,51	0.4162
MaxMoist	1.35	2,51	0.2506	2.20	2,51	0.1445	24.35	2,51	0.0001
IMIxMaxMoist	3.61	3,51	0.0629	0.02	3,51	0.8858	0.67	3,51	0.4162

[a] R-square of the model on data transformed to arcsine √proportion.

as soil moisture increased. Imidacloprid, *M. anisopliae,* and soil moisture had significant effects on larval mortality and mycosis, according to factorial analysis (Table 6.4). The chemical/fungus interaction was also significant and synergism was expressed at 7 and 14% moisture levels.

CHAPTER 7
DISCUSSION

According to McCoy et al. (1995), imidacloprid appeared to be systemic at doses of 200 ppm, and affected development of first instar larvae of *D. abbreviatus* by oral exposure at doses of 100 ppm or greater. Our studies confirmed these findings. In addition, we showed that larvae stopped feeding by oral exposure at doses as low as 12.5 ppm. Imidacloprid affected larval mobility and development by contact exposure at doses greater than 100 ppm. Larval mobility was significantly impaired in soil treated with imidacloprid at doses greater than 6μg/g soil. However, at a higher soil moisture level (12%), larval movement was significantly impaired even at 6μg/g soil. In terms of larval mortality, contact exposure in and outside a soil substrate at doses less than 500 ppm had little effect. However, larvae fed carrot or artificial diet were highly susceptible (100% mortality) at doses greater than 100 ppm, suggesting that the 6-fold difference in susceptibility was influenced by chemical penetration of the larval cuticle. Liu et al. (1993) showed that low contact toxicity by imidacloprid was due to poor cuticular penetration and rapid oxidative detoxification. When imidacloprid was applied as a soil drench to container-grown citrus trees at 200 ppm, no larval survival occurred in the soil. Since larval mortality in treated soil was low at doses less than 500 ppm, our study suggests that imidacloprid was systemic in citrus trees and toxic to larvae at low doses.

These data also showed that the mode of action for imidacloprid is slow for either

mode of entry. For example, 9 d were required to kill 70% of the larvae at 500 ppm by oral exposure and 1000 ppm was required to kill 50% by contact exposure. This slow mode of action is typical of some neurotoxins and has been reported for imidacloprid against the subterranean termite, *Reticulitermes flavipes* (Zeck and Monke 1992) and larvae of *Heliothis virescens* and *Spodoptera littoralis* (Lagadic and Bernard 1993). Although we did not quantify feeding behavior, larvae fed either carrot or synthetic diet exhibited reduced mobility and feeding activity. The latter likely contributed to slower development (ecdysis) since 14±5% of larvae without food molted compared with 80±6.3% of larvae with food. No reports of molting inhibition have been reported in the literature for imidacloprid. Antifeedant action caused by imidacloprid has been reported for *R. flavipes* (Zeck and Monke 1992), false wireworm, *Somaticus* spp (Drinkwater 1994), and black maize beetle, *Heteronychus arator* (Drinkwater and Groenewald 1994). However, Lagadic and Bernard (1993) found no evidence of antifeedant action or repellent effect with larvae of *H. virescens* and *S. littoralis* on artificial diet and concluded that restricted feeding was the result of intoxication involving paralysis or motor incoordination of the mouth parts. No paralysis of the larval mouthparts was observed in our study, in fact, excitation of the mouthparts occurred. We did observe abdominal tremors, uncoordinated quivering, erratic head movement, fluid discharge, and a tumbling effect, suggesting possible gut paralysis that would affect digestion of the food.

In our investigation, first instar larvae treated topically and orally were able to recover from imidacloprid treatment. Zeck and Monke (1992) and Boucias et al. (1996) demonstrated that imidacloprid induced significant behavioral changes in termites, however,

when tranferred to imidacloprid-free soil termites recovered from imidacloprid-induced paralysis. The nitromethylenes can be synergized by inhibitors of oxidative metabolism, cytochrome P450 inhibitors, suggesting that they undergo oxidative detoxification (Schroeder and Flattum 1984; Liu et al. 1993).

In chapter 3, we observed that loss of mobility in imidacloprid-treated larvae, prevented larvae from removing conidia upon contact with the surface of a substrate. Conidia were readily detached from fungus-only treated larvae as they crawled over the surface of the food source, the plastic container inner walls, and when actively moving through untreated soil. Boucias et al. (1988) observed that conidia of *M. anisopliae* were firmly attached to the cuticle of *Anticarsia gemmatalis* and several chemical treatments failed to remove significant number of attached conidia. In their studies, cuticle were obtained of larvae that were boiled for 2 h in a suspension containing SDS at 2% to remove all internal larval tissues. During this process, waxes, lipids, and other chemical compounds present on the epicuticule were also destroyed that probably facilitated strong attachment of conidia to the cuticle. In our studies, live insects were tested and the epicuticle was coated with continuous lipids and waxes secreted by the epidermal cells through lipids channels. This hydrophobic layer that provide a barrier to water loss, may also play an important role on attachment of infectious propagules.

Zacharuk (1970) demonstrated that conidia of *M. anisopliae* attached nonspecifically over the entire cuticle surface but were displaced from smooth sclerites of the epicuticle more easily than from the epicuticle folds. Our results also showed that conidia of *M. anisopliae* distributed uniformly over the entire cuticle surface of first instar larvae of *D.*

abbreviatus treated and untreated with imidacloprid. Conidia tended to attach to cuticular folds, around sensilla organelles, and on depressions present on larval epicuticle. Conidia were also observed on setae, around setal socket, and spiracle regions.

Cuticular exposure of larvae to formulated imidacloprid affected negatively conidial adhesion of *M. anisopliae*. Comparative bioassay data on the effect of the different components of imidacloprid on the attachment of conidia on larval cuticle showed that the inert carrier in the formulation was responsible for lower conidial attachment. However, in the soil, formulated imidacloprid significantly increased conidial attachment of both *M. anisopliae* and *B. bassiana* on larval cuticle. Conidial attachment was higher in soil treated with imidacloprid compared to control.

Both empirical and microscopic studies conducted in chapter 4 clearly showed that formulated imidacloprid, particularly certain components of the inert carrier stimulated the rate and amount of conidial germination of *M. anisopliae* and *B. bassiana*. Numerous research reports have shown that both carbon and nitrogen will stimulate conidial germination of both *M. anisopliae* and *B. bassiana* (Smith and Grula 1981, Dillon and Charnley 1990, Li and Holdom 1995). Component A is a trihydroxypropane containing 39.12% carbon obtained from fats and oils as by products in the manufacture of fatty acids. It is a humectant readily absorbing moisture from air and also H_2S, HCH, and SO_2. Moisture is vital to the germination of conidia causing hydrophobic forms commom to both *M. anisopliae* and *B. bassiana* (Ferron 1977, Roberts and Campbell 1977, McCoy et al 1988). Therefore, it is likely that the stimulation of conidial germination shown for component A is the result of both nutritional and physical factors expressing themselves on the artificial

substrates and larval cuticle. Component A is also a solvent, so it is conceivable that it breaks down the cuticular waxes on the cuticle making it more amenable to infection. The wax layer of the epicuticle have been reported to inhibit germination of entomopathogenic fungi (Smith and Grula 1982, Saito and Aoki 1983). Further research is required to confirm this hypothesis.

The active ingredient at 1,000 and 10,000 ppm also enhanced germination. Imidacloprid contains nitrogen that might be responsible for stimulating conidial germination. Li and Holdom (1995) found that conidia of *M. anisopliae* will germinate without an exogenous carbohydrate source in both liquid and solid media containing only KNO_3 as a nitrogen source.

Various bioassays performed in chapter 5 with different concentrations of either *B. bassiana* or *M. anisopliae* and different sublethal concentrations of imidacloprid as a contact or oral treatment, resulted in a pronounced synergistic increase in both mortality and mycosis of first instar larvae of *D. abbreviatus*. Synergism appeared temporal, in that chemical/fungal combinations reduced the time to mortality compared to single treatments and agrees with Benz (1971). Synergism was observed at concentrations of imidacloprid applied as contact exposure at doses of 100 ppm or greater. Below this concentration, larval mortality and LT50 values were similar to imidacloprid alone, because larvae recovered from chemical-induced paralysis in less than 24 h on contact exposure to imidacloprid at doses below 100 ppm, allowing time for conidial removal from the cuticle before germination and penetration. Similar results were observed by Boucias et al. (1996) working with the subterranean termite *Reticulitermes flavipes.* Termites treated with imidacloprid lost their

ability to remove conidia from each other cuticle during social grooming.

Although imidacloprid affected larval ecdysis negatively, failure of larvae to molt had no apparent effect on synergism. Most insects began molting within 3-4 days after treatment. Normally conidia attached to the cuticle before penetration are removed during ecdysis (Boucias and Pendland 1991, Hassan et al. 1989). Since the fungi used in these studies were only moderately virulent to the first instar larvae of *D. abbreviatus*, possibly germination was slow, allowing time for conidial removal by the larvae (See chapter 3). Copious conidial residues were detected on the food source and the inner wall of the plastic cups using plating on selective media. In addition, no fungus growth or conidia were observed on exuviae of fungus- treated larvae held in high humidity chambers.

Interaction of either *M. anisopliae* or *B. bassiana* with concentrations of imidacloprid greater than 100 ppm did not result in higher larval mycosis. Mycosis was actually reduced when imidacloprid was used at 1000 ppm. The lower conidial attachment on larvae treated with imidacloprid by contact exposure at doses greater than 100 ppm probably resulted in less larval mycosis. In chapter 3, I showed that component(s) present in the inert carrier affected negatively conidial adhesion on the larval cuticle when formulated product was tested at doses greater than 100 ppm. Further studies need to be conducted to determine the factor (s) associated with the inert carrier responsible for less conidial attachment.

In all bioassays conducted in chapter 6, soil application of different concentrations of conidia of either *M. anisopliae* or *B. bassiana* as an oil or wettable powder formulation had no effect on the movement of larvae of *D. abbreviatus* in soil at different moistures. One can speculate that larvae contracting fungal conidia on the soil surface will remove most

conidia when exposed to untreated soil. In chapter 3, I observed that healthy larvae actively moving through untreated soil remove most of the conidia from the cuticle. In addition, field studies showed that when *M. anisopliae* or *B. bassiana* conidia were applied to the surface of sandy soil common in citrus groves, inoculum gradually descend vertically or laterally over time (McCoy, unpublished data). In clay soils, however, movement of conidia were restricted to the upper 1-2 cm of soil (Storey and Gardner 1988, Storey et al. 1989, Villani et al. 1994).

The addition of imidacloprid to the soil significantly impaired larval movement. When the chemical was applied with a fungus, however, the effect on larval mobility was greater. Although imidacloprid impaired larval movement, it had little effect on larval mortality in soil. Similar findings were observed in other experiments performed in chapter 2. Larvae of *D. abbreviatus* also recovered after exposure to chemically treated soil and following oral and contact exposure to imidacloprid.

Soil treatment with unformulated conidia of *M. anisopliae* and *B. bassiana* as wettable powder at $5x10^3$, $5x10^4$, and $5x10^5$ caused minimal larval mortality of *D. abbreviatus*. In fact, larval mycosis (10.2%) was found only for the *B. bassiana* oil formulation at $5x10^5$ conidia/g soil. When sublethal doses of imidacloprid were combined with conidia of either fungus and applied to the soil, both larval mortality and mycosis increased synergistically. These results agree with those using a non-soil substrate reported in chapter 5. As discussed previously the retarded locomotory response of first instar larvae of *D. abbreviatus* treated with sublethal rates of imidacloprid is an important factor contributing to synergism between imidacloprid and entomopathogenic fungi. This

hypothesis is supported by the fact that in all bioassays, larval mortality and mycosis were highly correlated with larval mobility in soil. Higher levels of larval mortality and mycosis were always observed when fewer insects were recovered from Candler soil.

Movement of 48 h old larvae in sandy soil was not affected by soil moisture ranging from 2-12%, but they did not penetrate dry soil. Jones and Schroeder (1983) also found no soil penetration for first instar larvae of *D. abbreviatus* in dry soil, but they were successful when the moisture was between 7 and 16.7%. Movement of 48 h old larvae were retarded as moisture and soil depth increased. Newly-hatched larvae exhibited poor vertical movement suggesting that larval age is an important factor to consider in experimentation.

When conidia of *M. anisopliae* were combined with imidacloprid, larval mortality and mycosis decreased as moisture increased from 0 to 14%. Abundant mycelial growth was observed on cadavers held in soil at 0% water content but no conidia were visible. Several investigators have shown that *M. anisopliae* and *B. bassiana* can successfully infect host insects over a wide range of relative humidity, sometimes as low as 0% (Doberski 1981, Moore 1973, Ferron 1977, Ramoska 1984). Ramoska (1984) speculated that live insects have a boundary layer of moisture next to the cuticle, allowing germination of *B. bassiana* conidia on the host cuticle regardless of atmospheric relative humidity. Similar results were obtained by Krueger el al. (1991) and by Studdert and Kaya (1990), where they found that a proportionately greater percentage of chinch bugs, *Blissus leucopterus leucopterus* and the soybean caterpillar, *Spodoptera exigua*, became infected by *B. bassiana* when exposed to drier soils compared to wetter soils. Soil microorganisms that suppress germination of pathogenic fungi and reduce infection has been documented with insect and plant pathogens

(Clerk 1969, Smith and Snyder 1972, Hornby 1978, Marois and Mitchell 1981, Shields et al. 1981). In addition, certain microorganisms associated with the epicuticular surface, may influence conidial germination. Schabel (1976) reported that the presence of bacteria and fungi on *Hylobius pales* inhibited germination of *M. anisopliae* conidia. Most extensive microbial (mostly bacteria) decomposition of organic material occurs in wetter soils and there is little activity in drier soils (Sommers et. al 1981, Wilson and Griffin 1975). Probably, in higher soil moisture antagonistic microorganisms affected germination of *M. anisopliae* and fewer insects were infected by the fungus and/ or conidia were destroyed by soil microoorganisms.

Imidacloprid enhanced larval mortality and mycosis in two ways: 1) Temporary larval paralysis by the active ingredient that prevented the larvae from removing fungal conidia; 2) enhanced germination of fungal conidia by the inert carrier. Germination, however, works independently and must depend on the active ingredient to cause temporary larval paralysis, preventing conidial loss by the larvae.

Component A of the inert carrier, a stimulator of rate and amount of conidia germination of both *M. anisopliae* and *B. bassiana,* may be alone a potential synergist of the infection process for control of soil and above ground insects. This is probably true for both above and below ground insect pests that remains immobile or have little mobility sometime in their life cycle or can not void their cuticle of conidia. For soil insects, enhanced control by addition of a nutrient source may be feasible if antagonists do not overwelm fungal hyphae causing lyse or fungistasis (Cook and Baker 1983, Lingg and Donaldson 1981).

Employment of fungi for control of soil and above ground insects has declined over

the past decade due to requirement for high inoculum rates to achieve satisfactory level of insect control at field (McCoy 1990, Schwarz, 1995). In addition, overall performance of entomopathogenic fungi have been inconsistent at field (Villani 1992). The combination of sublethal doses of chemicals with entomopathogenic fungi may offer a new strategy for insect control where fungi can be used at cost-effective rates. Preliminary field studies on the combined effect of imidacloprid with *B. bassiana* for control of *D. abbreviatus* larvae in the soil suggest this is true. This biological/chemical synergism present an opportunity to reduce pesticide loads in the environment without sacrificing protection.

CHAPTER 8
CONCLUSIONS

1. Larvae fed imidacloprid-treated carrot or artificial diet at doses of 12.5 ppm and greater, fed less contributing to slower larval development and reduced ecdysis.

2. Imidacloprid by contact exposure at doses greater than 100 ppm affected larval mobility and development.

3. Time to larval mortality from imidacloprid was six times faster by oral compared to contact exposure.

4. Larval mobility was significantly impaired but had minimal effect on larval mortality in soil treated with imidacloprid at doses greater than 6 μg/g soil.

5. In moistened soil, larval movement was impaired significantly by imidacloprid even at 6 μg/g soil.

6. Following contact exposure, larvae recovered from imidacloprid-induced paralysis.

7. Subsequent to oral exposure to imidacloprid-treated carrot for 3 days, larval recovered showing no sluggishness or quivering behavior when placed on untreated carrot.

8. No larvae survived in the soil when imidacloprid was applied at 200 ppm as a soil drench to container-grown citrus trees suggesting systemic effect on the larvae.

9. Imidacloprid-treated larvae removed significantly fewer conidia from the cuticle during vertical and horizontal movement on different substrates compared to untreated larvae.

10. Conidial attachment of *M. anisopliae* to the larval cuticle was reduced when treated with imidacloprid at doses greater than 100 ppm.

11. The inert carrier was responsible for lower conidial attachment of *M. anisopliae* on larval cuticle.

12. In soil, formulated imidacloprid increased conidial attachment of both *M. anisopliae* and *B. bassiana* on larval cuticle.

13. Rate and amount of conidial germination of *M. anisopliae* increased 4 fold in time with an increase in imidacloprid at 1-10,000 ppm on water agar media.

14. Imidacloprid residue on the larval cuticle stimulated the rate and amount of conidia germination of *M. anisopliae* and *B. bassiana* following exposure to soil treated with imidacloprid at 25 μg/g soil.

15. Conidial germination and germ tube penetration through the cephalic capsule by *M. anisopliae* occurred within 12 hr after cuticular exposure to imidacloprid at 200 ppm.

16. Two components of the inert carrier, component A and to a lesser extent component B, stimulated conidial germination of *M. anisopliae*.

17. Technical imidacloprid at 1,000 and 10,000 ppm stimulated conidial germination of *M. anisopliae.*

18. The interaction of *Beauveria bassiana* or *Metarhizium anisopliae* with sublethal doses of imidacloprid as a contact or oral treatment, synergistically increased mortality and mycosis of first instar larvae of *Diaprepes abbreviatus* exclusive of soil.

19. Synergism occurred at concentrations of imidacloprid of 100 ppm or greater, when larval mobility was impaired.

20. The time to larval death was reduced significantly by the fungal/chemical treatment.

21. At fungal concentration of 10^6 and 10^7 conidia/ml and imidacloprid at doses of 100 ppm or greater, larval mortality and mycosis reached 90-100%.

22. Larval mycosis was reduced at doses of imidacloprid greater than 1000 ppm.

23. Larval mortality and mycosis by *M. anisopliae* was the same when imidacloprid was administered by contact or *per os.*

24. The active ingredient appeared to be an important component of the formulated product responsible for synergism through reduced mobility from neuro-paralysis.

25. Conidia of either *M. anisopliae* or *B. bassiana* applied to the soil as unformulated or an oil or wettable powder formulations had no effect on larval movement and mortality at different moistures.

26. Sublethal doses of imidacloprid in combination with conidia of either *M. anisopliae* or *B. bassiana* applied to the soil as an oil or wettable powder formulations significantly impaired larval movement and increased both larval mortality and mycosis in soil .

27. Vertical movement of 48 h old larvae in sandy soil required moisture.

28. Movement of 48 h old larvae were retarded as moisture and soil depth increased.

29. Mortality and mycosis of larvae by *M. anisopliae* alone were significantly higher at 0% compared to 7 and 14% moisture.

30. Larval mortality and mycosis decreased as moisture increased from 0 to 14% in soil treated with conidia of *M. anisopliae*/imidacloprid combination.

REFERENCES

Abbink, J. 1991. The biochemistry of imidacloprid. Pflanzenschutz-Nachrichten Bayer 44:183-195.

Al-Aidroos, K., and D. W. Roberts. 1978. Mutants of *Metarhizium anisopliae* with increased virulence towards mosquito larvae. Can. J. Gen. Cytol.20:211-219.

Anderson, T. E., A. E. Hajek, D. W. Roberts, H. K. Preisler, and J. L. Robertson. 1989. Colorado potato beetle (Coleoptera: Chrysomelidae): Effects of combinations of *Beauveria bassiana* with insecticides. J. Econ. Entomol. 82(1):83-89.

Beavers, J. B., C. W. McCoy, R. F. Kanaval, R. A. Sutton, and A. G. Selhime. 1972. The muscardine fungi pathogenic to *Diaprepes abbreviatus.* Fla. Entomol. 55(2):117-20.

Beavers, J. B., C. W. McCoy, and D. T. Kaplan. 1983. Natural enemies of subterranean *Diaprepes abbreviatus* (Coleoptera: Curculionidae) larvae in Florida. Environ. Entomol. 12(3):840-843.

Beilharz, V. C., D. G. Parberry, and H. J. Swart. 1982. Dodine: A selective agent for certain soil fungi. Trans. Br. Mycol. Soc. 79:507-511.

Benz, G. 1971. Synergism of micro-organisms and chemical insecticides, pp. 327-355. *In* H. D. Burges and N. W. Hussey [eds.] Microbial Control of Insects and Mites. Academic Press, New York, N.Y.

Boucias, D. G., and J. C. Pendland. 1991. Attachment of mycopathogens to cuticle. The initial event of mycoses in arthropod hosts, pp. 101-127. *In* G. T. Gole and H. C. Hoch [eds.] The Fungal Spore Disease Initiation in Plants and Animals. Plenum Publishing Corp., New york, N.Y.

Boucias, D. G., J. C. Pendland, and J. P. Latge. 1988. Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. Appl. Environ. Microbiol. 54:1795-1805.

Boucias, D. G., Stokes, C., Storey, G., and J. C. Pendland. 1996. The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana.* Pflanzenshutz-Nachrichten Bayer 49(2): 105-151.

Bullock, R. C. 1985. Potential for controlling citrus root weevil larvae and adults with chemicals. Fla. Entomol. 68(3):417-23.

Clerk, G. C. 1969. Influence of soil extracts on the germination of conidia of the fungi *Beauveria bassiana* and *Paecilomyces farinosus.* J. Invertebr. Pathol. 13:120-4.

Cook, R. J., and K. F. Baker. 1983. The nature and practice of biological control of plant pathogens. American Phytopathological Society, St. Paul, MN. 539 pp.

Daoust, R. A., and D. W. Roberts. 1982. Virulence of natural and insect-passaged strains of *Metarhizium anisopliae* to mosquito larvae. J. Invertebr. Pathol. 40:107-117.

Dillon, R. J., and Charnley, A. K. 1985. A technique for accelerating and synchronising germination of conidia of the entomopathogenic fungus *Metarhizium anisopliae.* Arch. Microbiol. 142:204-206.

Dillon, R. J., and Charnley, A. K. 1990. Initiation of germination in conidia of the entomopathogenic fungus, *Metarhizium anisopliae.* Mycol. Res. 94(3)299-304.

Doberski, J. W. 1981. Comparative laboratory studies on three fungal pathogens of the elm bark beetle Scolytus scolytus: Effect of temperature and humidity on infection by *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus.* J. Invertebr. Pathol. 37:195-200.

Drinkwater, T. W. 1994. Comparison of imidacloprid with carbamate insecticides, and the role of planting depth in the control of false wireworms, *Somaticus* species, in maize. Crop protection 13(5): 341-345.

Drinkwater, T. W., and L. H. Groenewald 1994. Comparison of imidacloprid and furathiocarb seed dressing insecticides for the control of the black maize beetle, *Heteronychus arator* Fabricius (Coleptera: Scarabaeidae), in maize. Crop Protection 13(6): 421:424.

Elbert, A., B. Becker, J. Hartwig, and C. Erdelen. 1991. Imidacloprid - a new systemic insecticide. Pflanzenschutz-Nachrichten Bayer 44:113-136.

Fargues, J. 1973. Sensibilite des larves de *Leptinotarsa decemlineata* Say (Col., Chrysomelidae) a *Beauveria bassiana* Vuill. (Fungi imperfecti, Moniliales) en presence of doses reduites d'insecticide. Ann. Zool. Ecol. Anim. 5:231-246.

1975. Etude experimentale dans la nature de l'utilisation combinee de *Beauveria bassiana* et d'insecticides a dose reduite contre *Leptinotarsa decemlineata.* Ann. Zool. Ecol. Anim. 7: 247-264.

1984. Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity, pp. 90-110. *In* J. Aist and D. W. Roberts [eds.] Infection Process of Fungi. Rockefeller Foundation Study Center, Bellagio, Italy.

Fargues, J., Reisinger, O., Robert, P. H., and C. Aubert. 1983. Biodegradation of entomopathogenic hyphomycetes: Influence of clay-coating on *Beauveria bassiana* blastospore survival in soil. J. Invertebr. Pathol. 41:131-142.

Fargues, J., and P. H. Robert. 1985. Persistance des conidiospores des hyphomycetes entomopathogenes *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sor., *Nomuraea rileyi* (F.) Samson et *Paecilomyces fumosoroseus* Wize dans le sol, en conditions controlees. Agonomie 5:73-80.

Ferron, P. 1977. Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* (Fungi Imperfecti, Moniliales) in imagines of *Acanthoscelides obtectus* (Col.: Bruchidae). Entomophaga 22:393-396.

Futch, S. and C. W. McCoy. 1994. Citrus root weevils. Florida Cooperative Extension Service, IFAS, University of Florida, Gainesville. SP 157:1-7.

Fuxa, J. R. 1987. Ecological considerations for the use of entomopathogens in IPM. Ann. Rev. Entomol. 32:225-251.

Gaugler, R., Costa, S. D., and J. Lashomb. 1989. Stability and efficacy of *Beauveria bassiana* soil inoculations. Environ. Entomol. 18:3:412-417.

Goral , V. M., and N. V. Lappa. 1974. The effect of beauverine and some pesticides on Colorado potato beetle populations. Zakhyst Rosl. Resp. Mizhvid. Temat. Nauk. Zb. 20:51-60.

Grodowitz, M. J., Krchma, J., and A. B. Broce. 1982. A method for preparing soft bodied larval Diptera for scanning electon microscopy. J. Kansas Entomol. Soc. 55(4):751:753.

Hall, D. G. 1995. A revision to the bibliography of the sugarcane rootstalk borer weevil, *Diaprepes abbreviatus* (Coleoptera: Curculionidae). Fla. Entomol. 78(2): 364-377.

Hallam, N. D. 1970. Leaf wax fine structure and ontogeny in eucalyptus demonstrated by means of a specialized fixation technique. J. Microsc. 92:137-144.

Hamlen, R. A., and J. B. Beavers. 1975. Toxicity and persistence of surface applied and soil incorporated insecticides against *Diaprepes abbreviatus* larvae. J. Econ. Entomol. 72:131-4.

Hassan, A. E. M., and A. K. Charnley. 1989. Ultrastructural study of the penetration by *Metarhizium anisopliae* through dimilin affected cuticle of Manduca sexta. J. Invertebr. Pathol., 54:117-124.

Hassan, A. E. M., R. J. Dillon, and A. K. Charnley. 1989. Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta*. J. Invertr. Pathol. 54: 277-279.

Hornby, D. 1978. Microbial antagonisms in the rhizosphere. Ann. Appl. Biol. 89:97-100.

Hung, S. Y. and D. G. Boucias. 1992. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua.* J. Invertebr. Pathol. 60:152-158.

Jones, I. F., and W. J. Schroeder. 1983. Study of first instar *Diaprepes abbreviatus* (Coleoptera: Curculionidae) activity for control purposes. J. Econ. Entomol. 76:567-569.

Keller, S. 1978. Investigations of the influence of Dimilin (Diflubenzuron) on growth and germination of conidia of some insect pathogenic fungi. Anz. Schaedlingskde. Pflanz-Umweltschutz, 51:81-83.

Keller, S. and G. Zimmermann. 1989. Mycopathogens of soil insects, pp.240-269. *In* Wilding, N., Collins, N., Hammond, N. M., Webber, P.M., and J. F. Webber [eds.]. Insect Fungus-Interactions. Academic Press, London.

Kendrick, W. B. and D. Parkinson. 1990. Soil fungi, pp.49-68. *In* D. L. Dindal [ed] Soil Biology Guide. John Wiley & Sons, New York, N. Y.

Krueger, S. R., Villani, M. G., Nyrop, J. P., and D. W. Roberts. 1991. Effect of soil environment on the efficacy of fungal pathogens against scarab grubs in laboratory bioassays. J. Biol. Control 1:203-209.

Krueger, S. R., Nechols, J. R., and W. A. Ramoska. 1991. Infection of chinch bug, *Blissus leucopterus leucopterus* (Hemiptera: Lygaeidae), adults from *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) conidia in soil under controlled temperature and moisture conditions. J. Invertebr. Pathol. 58: 19-26.

Lagadic, L., and L. Bernard. 1993. Topical and oral activities of imidacloprid and cyfluthrin against susceptible laboratory strains of *Heliothis virescens* and *Spodoptera littoralis*. Pestic. Sci. 38: 323-328.

Lacey, C. M., A. L. Lacey, and D. W. Roberts. 1988. Route of invasion and histopathology of *Metarhizium anisopliae* in *Culex quinquefasciatus*. J. Invertebr. Pathol. 52:108-118.

Li, D. P., and D. G. Holdom. 1993. Effect of soil matric potential on sporulation and conidial survival of *Metarhizium anisopliae* (Deuteromycotina:Hyphomycetes). J. Invertebr. Pathol. 62:273-277.

Li, D. P., and D. G. Holdom. 1995. Effects of nutrients on colony formation, growth, and sporulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). J. Invertebr. Pathol. 65:253-260.

Lingg, A. J., and M. D. Donaldson. 1981. Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. J. Invertebr. Pathol., 38:191-200.

Liu, M.-Y., and J. E. Casida. 1993. High affinity binding of [^{3}H]imidacloprid in the insect acetylcholine receptor. Pest. Biochem. Physiol. 46: 40-46.

Liu, M.-Y., J. Lanford, and J. E. Casida. 1993. Relevance of [^{3}H]imidacloprid binding site in house fly head acetylcholine receptor to insecticidal activity of 2-nitromethylene- and 2-nitroimino-imidazolidines. Pest. Biochem. Physiol. 46: 200-206.

Marois, J. J., and D. J. Mitchel. 1981. Effects of fungal communities on the pathogenic and saprophytic activities of *Fusarium oxysporium f. sp. radicis-lycopersici.* Phytopathology 71:1251-1256.

McCoy, C. W. 1990. Entomogenous fungi as microbial pesticides. *In* R. R. Baker, and P. E. Dunn [eds.], UCLA Symposia on Molecular and Cellular Biology, New Series. Vol. 112, pp 139-161.

McCoy, C. W. 1991. Survival of *Beauveria bassiana* as biological control agent in tropical soils. Achievements in Tropical and Subtropical Agricultural Research. Caribean Basin Administrative Group. St. Thomas, U.S. V. I. P. 45-47.

McCoy, C. W. and S. E. Simpson. 1994. Past and current IPM strategies to combat the spread of *Diaprepes abbreviatus* in Florida citrus. Proc. Caribbean Food Crops Soc. 30: 247-56.

McCoy, C. W., Samson, R. A., And D. G. Boucias. 1988. Entomogenous fungi. pp.151-236. *In* C. M. Ignoffo and N. B. Mandava, eds. Handbook of Natural Pesticides, Vol. V. Microbial pesticides, Part A. Entomogenous Protozoa and Fungi. CRC Press, Inc., Boca Raton, FL.

McCoy, C. W., and E. D. Quintela. 1994. Influence of the soil environment on entomopathogenic fungi as microbial control agents, p.47. *In* 4º. Simposio de Controle Biologico, Gramado, RS, Brazil.

McCoy, C. W., E. D. Quintela, S. E. Simpson, and J. Fojtik. 1995. Effect of surface-applied and soil-incorporated insecticides for the control of *Diaprepes abbreviatus* larvae in container-grown citrus. Proc. Fla. State Hortic. Soc. 108:130-136.

McCoy, C. W., Segretain, C., Beavers, G. M., and C. Tarrant. 1985. Laboratory rearing and some aspects of the biology of Artipus floridanus Horn (Coleoptera: Curculionidae). Fla. Entomol. 68(3):379-85.

Moore, G. E. 1973. Pathogenicity of three entomogenous fungi to southern pine beetle at various temperatures and humidities. Environ. Entomol. 2:54-57.

Osborne, L. S., and D. G. Boucias. 1988. A review of chemical antagonists to mycopathogens of citrus root weevils. Fla. Entomol. 68(3):409-16.

Quintela, E. D., Lord, J. C., Alves, S. B. and D. W. Roberts. 1992. Persistencia de *Beauveria bassiana* em solo do cerrado e sua interação com microorganismos do solo. An. Soc. Entomol. Brasil 21:1:69-80.

Quintela, E. D., Wraight, S. P., Quindere, M. A. W., and D. W. Roberts. 1994. Aplicação de conídios de *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sor. Para controle de larvas de *Chalcodermus bimaculatus* Boh. (Coleoptera: Curculionidae) no solo. An. Soc. Entomol. Brasil 23:3:367-377.

Ramoska, W. A. 1984. The influence of relative humidity on *Beauveria bassiana* infectivity and replication in the chinch bug, *Blissus leucopterus.* J. Invertebr. Pathol. 43:389-394.

Roberts, D. W., and A. S. Campbell. 1977. Stability of entomopathogenic fungi. Miscell. Pub. of the Entomol. Soc. Am. 10(3):19-76.

Saito, T., and Aoki, J. 1983. Toxicity of free fatty acids on the larval surfaces of two lepidopterous insects towards *Beauveria bassiana* (Bals.) Vuill. And *Paecilomyces fumoso-roseus* (Wize) Brown et Smith (Deuteromycetes: Moniliales). Applied Entomology and Zoology 18:225-233.

Samuels, K. D. Z., Heale, J. B., and M. Llewellyn. 1989. Characteristics relating to the pathogenicity of *Metarhizium anisopliae* toward *Nilaparvata lugens.* J. Invertebr. Pathol. 53:25-31.

SAS Institute. 1985. SAS User's Guide: Statistics, version 5 ed. SAS Institute, Cary, NC.

Schabel, H. G. 1976. Green muscardine disease of *Hylobius pales* (Herbst) (Coleoptera: Curculionidae). Z. Ang. Entomol. 81:413-421.

Schroeder, M. E., and R. F. Flattum. 1984. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. Pest. Bioch. Physiol. 22: 148-160.

Schwarz, M. R. 1995. *Metarhizium anisopliae* for soil pest control. *In* Biorational Pest Control Agents: Formulation and Delivery. F. R. Hall and John Barry (eds.) Proc. Amer. Chem. Soc. pp. 153-196

Shields, M. S., Lingg, A. J., and R. C. Heimsch. 1981. Identification of a *Penicillium urticae* metabolite which inhibits *Beauveria bassiana.* J. Invertebr. Pathol. 38:374-377.

Smith, R. J., and E. A. Grula. 1981. Nutritional requirements for conidial germination and hyphal growth of *Beauveria bassiana.* J. Invertebr. Pathol. 37:222-230.

Smith, S. N., and W. C. Snyder. 1972. Germination of *Fusarium oxysporium* chlamydospores in soils favorable to wilt stablisment. Phytopathology 62:273-277.

Sommers, L. E., Gilmour, C. M., Wildung, R. E., and S. M. Beck. 1981. The effect of water potential on decomposition processes in soils, pp.97-117. In Water Potential Relations in Soil Microbiology, Soil Sci. Soc. Am. Spec. Publ., 9.

Sosa-Gomez, D. R., D. G. Boucias, and J. L. Nation. 1996. Attachment of *Metarhizium anisopliae* to the southern green stink bug *Nezara viridula* cuticle and fungistatic effect of cuticular lipids and aldehydes. J. Invertebr. Pathol. (In press).

Steinhaus, E. A. 1956. Microbial control- the emergence of an idea. A brief history of insect pathology through the nineteenth century. Hilgardia 26:107-160.

Storey, G. K., and W. A. Gardner. 1988. Movement of an aqueous spray of Beauveria bassiana into the profile of four Georgia soils. Environ. Entomol. 17(1): 135-139.

Storey, G. K., Gardner, W. A., and Tollner, E. W. 1989. Penetration and persistence of commercially formulated *Beauveria bassiana* conidia in soil of two tillage systems. Environ. Entomol. 18(5):835-839.

Studdert, J. P., and H. K. Kaya. 1990. Water potential, temperature, and clay-coating of *Beauveria bassiana* conidia: Effect on *Spodoptera exigua* in two soil types. J. Invertbr. Pathol. 56:327-336.

Studdert, J. P., Kaya, H. K., and J. M. Duniway. 1990. Effect of water potential, temperature, and clay-coating on survival of *Beauveria bassiana* conidia in a loam and peat soil. J. Invertebr. Pathol., 55:417-427.

Sutton, R. M., Gardner, W. A., Kraus, D. W., and Noblet, R. 1979. Effects of ecdysone, juvenile hormone, and structurally-related compounds on *Nomuraea rileyi, Beauveria bassiana*, and *Tritirachium dependens* in vitro. J. Ga. Entomol. Soc. 14:364.

Telenga, N. A. 1968. On the effect of combined action of pathogenic microorganisms and insecticides upon insects. Zh. Obshch. Biol. 29:501-514.

Villani, M. G., Krueger, S. R., and J. P. Nyrop. 1992. Interaction of scarab grubs and microbial pathogens in the soil environment, pp. 11-126. *In* T. Jackson & T. Glare [eds.], Pathogens in Scarab Pest Management. Intercept, Andover, MA.

Villani, M. G., Krueger, S. R., Schroeder, P. C., Consolie, F., Consolie, N. H., Preston-Wilsey, L. M., and D. W. Roberts. 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. Environ. Entomol. 23:2:502-503.

Wilson, J. M., and D. M. Griffin. 1975. Water potential and the respiration of microorganisms in the soil. Soil Biol. Biochem., 7, 199-204.

Woodruff, R. E. 1964. A Puerto Rican weevil new to the United States (Coleoptera: Curculionidae). Fla. Dept. Agric., Div., Plant Ind. Entomol. Circ. 30: 1-2.

1985. Citrus weevils in Florida and the West Indies: Preliminary report on systematics, biology, and distribution (Coleoptera: Curculionidae). Fla. Entomol. 68: 370-379.

Wraight, S. P. and D. W. Roberts. 1987. Insect control efforts with fungi. J. Ind. Microbiol. 28:77-87.

Zacharuk, R. Y. 1970. Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae (Coleoptera). J. Invertebr. Pathol. 15:63-80.

Zeck, W. M. 1992. Synergism between a new insecticide and entomopathogenic fungi in the control of subterranean termites. 25th Ann. Meeting Soc. Invert. Pathol., Heidelberg, Germany.

Zeck, W. M., and B. J. Monke. 1992. Synergy in soil and bait applications of imidacloprid with Entomogenous fungi in termite control. ESA Annual Meeting 1992, Baltimore, MD.

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